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**AUTOCRINE MECHANISMS MODULATING ENDOCRINE
REGULATION OF MAMMARY GLAND FUNCTION**

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LIST OF ABBREVIATIONS

The abbreviations used here are those described as "accepted" in the instructions to authors of the *Biochemical Journal* (*Biochem. J.*, (1983). **233**, 1-24.), with the following additions.

CHAPS	- 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulphonate
cortisol	- hydrocortisone-21-acetate
d.f.	- degrees of freedom
FIL	- feedback inhibitor of lactation
IGF-I	- insulin-like growth factor I
IGF-II	- insulin-like growth factor II
IGFBP-3	- insulin-like growth factor binding protein 3
oPrl	- ovine prolactin
rBST	- recombinant bovine somatotrophin
RMYQ	- relative milk yield quotient
SEM	- Standard error of the means
WAP	- whey acidic protein

ABSTRACT

This thesis examines the mechanisms by which local changes in the frequency and efficiency of milk removal modify the regulation of the mammary gland by circulating galactopoietic hormones.

Individual mammary glands of a suckled, lactating rabbit were sealed to allow effects arising from engorgement of the gland with milk to be studied. Accumulation of milk in the mammary gland significantly reduced mammary prolactin receptor number, which was assessed in 4.5 M-MgCl₂ stripped microsomal membranes. This reduction in receptor number was apparent at the end of the normal (24 h) suckling interval and preceded the locally-induced decrease in the rate of milk accumulation previously shown to occur after this time. The effect of milk accumulation on hormone binding was due, at least in part, to the actions of the feedback inhibitor of lactation (FIL). This milk constituent, which is thought to be responsible for the local control of milk secretion, reduced both prolactin and IGF-I receptor number when introduced into the mammary gland via the teat duct.

In contrast, in the goat, more frequent removal of milk for 9 days did not affect prolactin receptor number, although it did stimulate the rate of milk secretion. In this case, it was possible that there was an effect on the distribution of receptors within the secretory cell.

The final part of this study investigated the mechanism of FIL action on hormone receptor number. For this purpose, methods were developed for the isolation and culture of mammary cells, by enzymic digestion of mid-lactation mouse mammary gland. Incubation of isolated cells with a 10-30 kDa goat whey fraction containing FIL, for 2 hours, resulted in prolactin receptors being relocated from cell-surface to intracellular sites, without affecting total receptor number. This FIL-induced reduction in hormonal sensitivity did not appear to mediate FIL's acute effects on protein secretion, since these were independent of exogenous prolactin during this short-term culture.

In conclusion, this study demonstrates local modulation of endocrine regulation of the lactating mammary gland. The effects of milk accumulation and alterations in milking frequency and efficiency on mammary prolactin receptor number and distribution are due, at least in part, to FIL. These FIL-induced changes in cellular sensitivity to circulating galactopoietic hormones could have important long-term effects on milk yield and mammary differentiation.

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DECLARATION

All data were collected by myself between 1989 and 1992, with the exceptions of the 5'-nucleotidase enzyme assay performed by Dr. S. Wastie (Chapter 3), SDS-gel electrophoresis performed by Professor W.L. Hurley (Chapter 3) and electron microscopy performed by Dr. K.A.K. Hendry (Chapter 6). With these exceptions, I declare that the work contained in this thesis is my own, undertaken under the supervision and guidance of Dr. C.H. Knight and Dr. C.J. Wilde. No part of this work has been submitted for consideration for any other degree or award.

Crispin N. Bennett

CHAPTER ONE

REVIEW OF THE LITERATURE

1.1 OVERVIEW

"Lactation is the final phase of the complete reproductive cycle of mammals. In almost all species the newborn are dependent on maternal milk during the neonatal period; in most the young are dependent for a considerable time. Adequate lactation is therefore essential for reproduction and the survival of the species and, biologically, failure to lactate can be just as much a cause of failure to reproduce as is failure to mate or to ovulate." (WHO Technical Report, 1965)

In view of the importance of lactation in ensuring successful reproduction, it is vital that the supply of maternal milk matches the needs of the neonate. Milk secretion by the mammary gland and therefore the supply of nutrients to the young is controlled at two levels, systemically and locally. During lactation, the level of nutritional investment by the mother is genetically programmed and modified both by nutritional status (Peaker, 1989) and by factors resulting from the suckling stimulus or, in dairy animals, the milking regime (see Cowie *et al.*, 1980). This systemic regulation of lactation is signalled to the mammary gland by the endocrine system and also acts to alter nutrient partitioning and physiological parameters such as cardiac output and voluntary food intake (see Vernon (1988) & Collier *et al.* (1984)).

The response to the systemic control of milk secretion is modulated locally by a mechanism which operates within each mammary gland. This local control of milk secretion responds to changes in both the frequency and the completeness of milk removal, matching the capacity for milk secretion to the rate of milk removal by the young or, in the case of dairy animals, the milking machine.

1.2 MAMMARY GLAND GROWTH AND DIFFERENTIATION

A satisfactory lactation can only be expected when the mammary glands have reached a proper state of development, both in terms of the number of cells and in their ability to synthesise and secrete milk. The majority of mammary gland growth occurs during pregnancy (Knight & Peaker, 1982a). The degree to which growth is complete at parturition and the relative contributions of cell number and secretory activity to milk yield throughout lactation varies between species (Knight & Peaker, 1982a). In mice, mammary cell number increases for the first 5 days of lactation

(Knight & Peaker, 1982b), with increases in cellular activity continuing until peak lactation on day 15 (Shipman *et al.*, 1987). In rats, cell proliferation may account for as much as three-quarters of the increase in daily milk yield leading up to peak lactation, with the remaining increase in yield being accounted for by greater cellular activity (Knight *et al.*, 1984). In the goat, by contrast, increasing milk yield is matched by increasing cell number over the first 3 weeks of lactation (Knight and Peaker, 1984), with enhanced cellular activity alone accounting for the further rise in milk yield to peak lactation (Wilde *et al.*, 1986).

Following peak lactation, the volume of milk produced by the mother gradually declines until such time as milk removal finally ceases. The maintenance of milk secretion during the later stages of lactation depends on the number of secretory cells lost, the extent of cell replacement (if any) and the retention of synthetic capacity by the cells. In the mouse, declining lactation is characterised by reductions in both the synthetic capacity of the cells and cell number (Shipman *et al.*, 1987), whilst in the rat decreasing cellular activity is entirely responsible for the decline in milk yield (Knight *et al.*, 1984). In the goat, in contrast, the decline in milk yield is primarily due to a decrease in secretory cell number (Wilde *et al.*, 1989b), with individual cells not losing their capacity for milk synthesis to any significant degree.

Cellular activity is largely determined by the degree of differentiation. Differentiation is classically defined as the process whereby the mammary epithelial cell acquires the complement of intracellular enzymes and proteins necessary to meet the prodigious demands of milk synthesis and secretion. Differentiated milk-secreting cells are histologically characterised by their large size and by the polarisation of membranous organelles, with abundant endoplasmic reticulum located basally and Golgi-derived vesicles in the apex of the cell. Evidence of secretory activity includes a highly folded apical membrane, fat droplets in the cytosol and casein micelles in secretory vesicles (reviewed by Pitelka & Hamamoto (1983)). The rate of milk component synthesis can be assessed in tissue explants (Wilde *et al.*, 1986), whilst levels (either as mRNA (Wilde *et al.*, 1990), or as the final protein (Speake *et al.*, 1975)) and activities of enzymes involved in both milk synthesis and flux control along metabolic pathways can also be used as markers of mammary differentiation (eg Wilde *et al.*, 1987b).

The mammary gland displays changing levels of differentiation throughout the lactation cycle (Wilde *et al.*, 1986; Wilde & Knight, 1989). These changes in the net differentiative state of the gland may reflect alterations in the proportion of secretory alveoli which are fully-differentiated at any one time, since gene expression in mammary tissue is apparently not homogeneous (Molenaar *et al.*, 1992). Alternatively, or in addition to the above, the level of differentiation of individual epithelial cells may change throughout lactation.

It is not known which population of mammary cells is responsible for the mammary growth that occurs during lactation i.e. does growth result from the proliferation of all non-differentiated cells (discussed by Knight & Peaker, (1982a)), or from the presence of a stem-cell population (Medina & Smith, 1990). It should be noted that the latter has not been conclusively demonstrated in the mammary gland. However, the dogma that proliferation and differentiation are mutually exclusive may be incorrect and it is possible that cells can dedifferentiate in order to proliferate, for histological observations have demonstrated the presence of secretory products in mitotic cells (Franke & Keenan, 1979; Traurig, 1967).

The factors controlling differentiation in the mammary gland have been studied extensively. Early studies *in vivo* demonstrated an effect of a combination of the hormones estrogen, progesterone, adrenal steroids, prolactin and growth hormone in inducing full lobuloalveolar development of the mammary gland in hypophysectomised- ovariectomised - adrenalectomised rats, with only prolactin and adrenal steroids being required for milk secretion (Lyons *et al.*, 1958). The effects of these hormones, with insulin, on the ultrastructure and differentiation of mammary epithelial cells has been confirmed *in vitro* (reviewed by Topper & Freeman (1980)). In addition, the importance of cell polarisation (Emerman & Pitelka, 1977) and cell-cell and cell-substratum interactions in mammary differentiation has also been demonstrated *in vitro* (reviewed by Howlett & Bissell (1990)).

1.3 MILK SYNTHESIS AND SECRETION

The function of the fully-differentiated mammary epithelial cell is the synthesis and secretion of milk. The major components of milk, apart from water, are lactose, fats and milk proteins.

1.3.1 Lactose and the control of lactose synthesis

In most species, lactose is the predominant milk carbohydrate and is involved in maintaining milk osmolarity. Lactose is synthesised, within the Golgi complex of mammary epithelial cells, by lactose synthetase. This enzyme consists of the ubiquitous galactosyl transferase and the specifier protein α -lactalbumin. The synthesis of lactose results in the osmotic entry of water into the secretory vesicles and this is why the rate of lactose synthesis dictates milk volume. The secretory vesicles, which also contain all the major organic components of skim milk, fuse with the apical membrane and release their contents into the milk by exocytosis. It takes approximately 1.5 hours for a lactose precursor to pass from blood to secreted milk (Linzell *et al.*, 1976). For more detailed accounts of lactose synthesis and secretion, the reader is referred to reviews by Kuhn (1983b), Mather and Keenan (1983), Mepham (1987) and Leong (1990).

Although lactose and other milk solids make their appearance in the gland well before the end of pregnancy (lactogenesis stage I, (Fleet *et al.*, 1975)), the major increase in the rate of lactose synthesis occurs around the time of parturition (lactogenesis stage II). This results in copious milk secretion and is tied in with the hormonal changes occurring at parturition. Progesterone has a direct inhibitory effect on lactogenesis and it is believed that withdrawal of progesterone together with an increase in circulating prolactin constitutes the lactogenic "trigger" (reviewed by Kuhn (1983a)).

In the mouse, maximal induction of α -lactalbumin mRNA requires prolactin and glucocorticoid in the presence of insulin, although this is overridden during pregnancy by progesterone (Kuhn, 1983b). In the pregnant rabbit, although local initiation of lactose (Chadwick, 1962) and milk synthesis (Bradley & Clarke, 1956) can be induced by injecting prolactin into the mammary gland via the teat canal, a control mechanism operates which is similar to that in the mouse (Cowie *et al.*, 1980). In marsupials by comparison, there is no inhibition from progesterone and maximal induction of α -lactalbumin mRNA is induced by prolactin alone (Collet *et al.*, 1990). Although important during the onset of lactose synthesis there is no evidence that α -lactalbumin is rate-limiting after this time, with lactose synthetase activity probably being governed by galactosyl transferase during early lactation (to day 16) (Wilde & Kuhn, 1979).

During lactation itself, the prime regulator of lactose synthesis is the concentration of glucose within the Golgi apparatus rather than the activity of the lactose synthetase enzyme (Faulkner & Peaker, 1987; Threadgold & Kuhn, 1984). Uptake of glucose across both the plasma and Golgi-enriched membranes of the lactating rat mammary gland is by a glucose transporter (Madon *et al.*, 1990), with transporter number correlating highly with the milk yield and being regulated in a coordinated manner by both prolactin and growth hormone, but not IGF-I (Fawcett *et al.*, 1991). Hormonal regulation of mammary glucose uptake by glucose transporter proteins offers a possible mechanism for the control of lactose synthesis and ultimately milk secretion. The arterial supply of glucose to the mammary gland is not a factor normally limiting lactose synthesis, but may be of importance when the animal is in negative energy balance (Linzell, 1967). For reviews on the control of lactose synthesis, the reader is directed to Vonderhaar (1989) and Kuhn (1983b).

1.3.2 Control of milk protein gene expression, synthesis and secretion

The mechanism of milk protein synthesis is essentially identical to that of all secreted proteins in eukaryotic cells. Milk protein genes are under transcriptional, translational and post-translational control by prolactin and other hormones. Once mRNA expression has been initiated, protein synthesis depends ultimately on a number of transcriptional conditions, such as the rate of transcription and the transcript half life. The majority of studies have centred on casein, the most abundant protein produced by the mammary gland, although the induction of milk proteins is not coordinately regulated and casein mRNA expression can occur in the absence of α -lactalbumin and whey acidic protein (WAP) mRNA expression *in vitro* (Lee *et al.*, 1985; Lee *et al.*, 1984). Effects on gene expression are chronically, rather than acutely, regulated, for although casein mRNA induction occurs within one hour of prolactin addition *in vitro*, marked increases in mRNA levels do not occur until several hours later (Matusik & Rosen, 1978). This induction is the consequence of an enhanced rate of gene transcription, with prolactin also acting to stabilise the synthesised transcript and increase the mRNA's half life (Teyssot & Houdebine, 1980; Teyssot & Houdebine, 1981).

Increased mRNA levels tend to result in an increased rate of protein synthesis (Houdebine & Gaye, 1975), although this is not always the case and in pseudopregnant rabbits the prolactin-induced increase in casein mRNA is not

accompanied by an equivalent stimulation of casein synthesis (Houdebine, 1976). In addition, lactating mice expressing foreign milk protein genes show an increased milk-protein synthesis *in vitro* which is not matched *in vivo* (Wilde *et al.*, 1992). It is apparent therefore that mRNA levels do not always limit milk protein synthesis and post-translational control of protein synthesis must be operating. The possible mechanisms for such control include hormonal regulation of translation through effects on ribosome concentration (Turkington & Riddle, 1970) and limitation by aminoacyl-tRNA synthetases or aminoacyl-tRNA availability (Devinoy *et al.*, 1978). Amino acid uptake by the mammary gland is regulated both by circulating prolactin and the local accumulation of milk (Vina *et al.*, 1981) and this could be an additional mechanism by which protein synthesis is controlled.

Following routing, together with lactose, through the Golgi apparatus and secretory vesicles (Mather & Keenan, 1983), milk protein secretion is by Ca^{2+} - dependent and independent pathways (Turner *et al.*, 1992a). The regulation of the Ca^{2+} -independent pathway by the feedback inhibitor of lactation (FIL) (Rennison *et al.*, 1993) will be discussed in detail at a later stage. The release of secretory vesicle contents at the apical cell surface is by exocytosis and can be stimulated by protein kinase C (Turner *et al.*, 1992b).

Caseins, once synthesised, are susceptible to intracellular degradation during secretion (Wilde *et al.*, 1991). A high proportion of casein is degraded in mammary explants prepared from pregnant animals, with little or no degradation being observed in explants from lactating animals (O'Hare *et al.*, 1986; Wilde & Knight, 1986). This process appears to be inversely related to the differentiative state of the secretory tissue (Stewart *et al.*, 1988; Wilde *et al.*, 1989a) and may act to modulate net protein secretion in mammary tissue from pregnant animals.

1.3.3 Milk fat and the control of fat synthesis

Fatty acids are synthesised in the cytoplasm of milk secreting cells by acetyl-CoA carboxylase and fatty acid synthetase, with palmitate ($\text{C}_{16:0}$) being the predominant fatty acid in milk. In the rabbit, thioesterase II is involved in the synthesis of medium chain ($\text{C}_{8:0}$ - $\text{C}_{12:0}$) fatty acids and is found exclusively in the mammary gland, with high levels in rabbits, rats and mice (Vonderhaar, 1987). Elaboration into triglycerides, which make up nearly all of the milk fat, takes place on the smooth endoplasmic reticulum and it is here that the fat droplets fuse prior to

secretion. Fat droplets are transported unidirectionally towards the apical surface and bud directly into the milk. The subject of fat synthesis is reviewed in Dils (1983), and the mechanism of lipid secretion is reviewed in Mather & Kennan (1983).

In the lactating rat, half of the mammary glands requirement for triacylglycerol is met from circulating lipoproteins, the remaining half from *de novo* synthesis within the gland. The rate of mammary lipogenesis is stringently regulated according to the level and type of substrate available to the gland (Munday & Hardie, 1987). In addition, medium-chain fatty acids, which are present in rat milk, inhibit mammary lipogenesis *in vitro* as a consequence of a direct inhibition of glycolysis (Heesom *et al.*, 1992). This acute regulation of milk fat synthesis may also operate *in vivo*, offering a mechanism of control in addition to that afforded by the modulation of the levels and activities of lipogenic enzymes.

Prolactin is involved in the long-term control of lipogenic enzymes, acting on sheep mammary explants to activate and increase the total level of acetyl-CoA carboxylase (Barber *et al.*, 1991), a key regulatory enzyme that catalyses the first reaction committed to fatty acid synthesis. Prolactin is also required for the induction of fatty acid synthetase (Speake *et al.*, 1975) and thioesterase II, as assessed by medium-chain fatty acid synthesis (Forsyth *et al.*, 1972; Speake *et al.*, 1975).

1.4 ENDOCRINE REGULATION OF THE MAMMARY GLAND

The endocrine control of the mammary gland varies greatly between species, but always acts to integrate the control of lactation with other aspects of reproduction, including parturition. The growth of the mammary gland is regulated throughout postnatal life by mammogenic hormones from the anterior pituitary, ovary and adrenal cortex and in many species these are elaborated during pregnancy by the placenta.

The following sections will describe the endocrine changes during pregnancy, parturition and lactation, concentrating mainly on prolactin, placental lactogen and growth hormone.

1.4.1 Hormone levels in pregnancy

Pregnancy creates a hormonal milieu that triggers extensive development of the mammary gland in preparation for lactation. In many species, placental lactogen is secreted by the placenta and is the major contributor to lactogenic activity during gestation (Forsyth, 1986). Placental lactogen levels are higher in animals carrying a large number of foetuses, resulting in a greater degree of mammary growth appropriate to the needs of the young (Flint *et al.*, 1985; Knight & Peaker, 1982c).

Goats and seasonally breeding sheep are pregnant throughout the winter and prolactin levels remain low until a few days before parturition. Placental lactogen is largely responsible for the lactogenic activity and levels of this hormone start to rise prior to mid-pregnancy and plateau between day 110 and parturition at day 150. The rising levels of prolactin at parturition offset the loss of placental lactogen from the system (Cowie *et al.*, 1980). In cows, the story is uncertain, for although bovine placental lactogen differs from and is present in the circulation at far lower levels than that found in other species (Bremel & Schuler, 1987), it has potent somatogenic, and possibly lactogenic, effects (Byatt *et al.*, 1991).

In rodents, coitus results in bi-daily prolactin surges which are replaced by rising placental lactogen levels. Both the mouse and the rat show different forms of placental lactogen between early and late pregnancy. In the rat, the late form of placental lactogen (from day 12) is involved in mammary gland development, with increasing levels in mid- to late- pregnancy which were shown, on day 21 (term), to increase exponentially with increasing foetus number (Robertson & Riesen, 1981).

In rabbits, prolactin levels rise in early pregnancy and then decrease before rising again prior to parturition. Although it is claimed that placental lactogen has been characterised from this species, comparison of prolactin and total lactogenic activity levels would suggest it is of minor physiological significance (Forsyth, 1986).

1.4.2 Parturition and lactogenesis

The mammary gland in the goat secretes low levels of a milk-like substance at about days 80-90 of gestation (lactogenesis stage I) (Fleet *et al.*, 1975). The subsequent onset of copious milk secretion (lactogenesis stage II) is dependent on systemic factors, in addition to local factors arising within the mammary gland.

Parturition results in a marked change in the hormonal milieu and is characterised by falling levels of progesterone and in some species placental lactogen.

At the same time, there is an increase in the circulating levels of prolactin, oestrogens, prostaglandin $F_{2\alpha}$, oxytocin and adrenal corticoids. Progesterone withdrawal at parturition, as a result of loss of placental support, acts together with adequate levels of lactogenic hormones and milk removal to stimulate the onset of copious milk secretion. During late pregnancy in goats, prostaglandin $F_{2\alpha}$ is synthesised within the mammary gland and between 3 to 5 days prepartum this secretion is switched from the blood to the milk. Prostaglandin $F_{2\alpha}$ is an active inhibitor of milk secretion and possibly has to be removed from the mammary gland, either by milk removal or metabolism, before copious milk secretion can start (Maule Walker, 1984).

1.4.3 Hormone levels in lactation

In the goat, both prolactin and growth hormone are released at milking. Prolactin release is reduced as a result of reduced day length as lactation progresses (Hart, 1975), with growth hormone release showing an increased milking response in late lactation (Hart & Flux, 1973).

In the rat, raising the serum prolactin concentration in late lactation can prevent the decline in milk yield (Flint *et al.*, 1984). There is little information on growth hormone levels during lactation in the non-ruminant. In mice, growth hormone levels rise throughout pregnancy, decreasing after parturition to basal levels before rising again (Sinha *et al.*, 1974), a pattern that is not reflected by circulating IGF-I levels (Travers *et al.*, 1990). In rabbits, there is a prolactin surge at suckling which is immediate and prolonged beyond suckling. In late lactation, the surge in prolactin concentration is more transient with a rapid return to basal levels (McNeilly & Friesen, 1978), with the suckling behaviour of the pups being implicated in this reduced release of prolactin (Mena *et al.*, 1990b).

1.5 ENDOCRINE REQUIREMENTS FOR LACTATION

1.5.1 Rats

As already described, Lyons (1958) demonstrated, using triply-operated rats, the minimum hormonal requirements for mammary gland growth, differentiation and milk secretion in this species. The role of prolactin in lactation can be clarified further by studies in which prolactin secretion is specifically inhibited by administration of the ergot-alkaloid bromocriptine (Cowie *et al.*, 1980). The actions

of growth hormone can be elucidated by neutralisation with specific antiserum (Madon *et al.*, 1986). During lactation in the rat, prolactin is the predominant galactopoietic hormone with its effects being modulated by growth hormone; treatment with both bromocriptine and anti-growth hormone sera being required to fully inhibit milk secretion (Madon *et al.*, 1986).

1.5.2 Goats

Prolactin is involved in the initiation of lactation in this species and inhibition of prolactin secretion at parturition suppresses the subsequent lactation, although not completely so (Akers *et al.*, 1981). Prolactin is required together with glucocorticoids, thyroid hormones and growth hormone to restore lactation fully following hypophysectomy (removal of the pituitary gland) (Cowie *et al.*, 1964).

The maintenance of an established lactation is termed galactopoiesis and is dependent upon continued milk removal and suitable hormonal support. Growth hormone is the predominant galactopoietic hormone in the goat (Cowie *et al.*, 1980). Prolactin is classically regarded as not being involved in galactopoiesis, as a result of early experiments in which milk yield was not affected following prolactin withdrawal in hypophysectomised animals, or once-daily bromocriptine-treatment in intact animals (Cowie *et al.*, 1964; Hart, 1973). However, thrice-daily bromocriptine treatment significantly reduces milk yield and increases the sensitivity of the mammary gland to unilateral more-frequent milking (Knight *et al.*, 1990a), whilst once-daily bromocriptine treatment in late-lactation affects lactational persistency (Gabai *et al.*, 1992). Other evidence for the involvement of prolactin in galactopoiesis is less clear. Intensification of the milking-induced prolactin release increased milk yield, although this was not statistically significant (Jacquemet & Prigge, 1991), whilst treatment with perphenazine, which has been reported to elevate circulating prolactin concentrations, greatly reduced the rate of milk yield decline (Vandeputte-Van Messom & Peeters, 1982). In the latter study however, an effect of perphenazine on levels of circulating growth hormone cannot be ruled out. In the goat, a working hypothesis would be that the milk yield of an individual gland is modulated by prolactin, within the systemic limits placed, albeit indirectly, on that gland by the actions of hormones such as growth hormone which affect metabolic partitioning (homeorhesis) within the whole animal (Bauman & Currie, 1980).

1.5.3 Rabbits

Prolactin initiates lactogenesis, although other hormones may be involved in the physiological preparation of the gland for prolactin action. Following hypophysectomy, prolactin and growth hormone alone produced a partial recovery of milk yield which was not augmented by adrenal steroids or adrenocorticotrophin (ACTH) (Cowie *et al.*, 1969; Hartmann *et al.*, 1970). Galactopoiesis is entirely prolactin dependent in this species, with milk secretion being abolished by the treatment of intact animals with the prolactin antagonist bromocriptine (Mena *et al.*, 1982; Taylor & Peaker, 1975).

1.6 ENDOCRINE ROLE OF THE MAMMARY GLAND

In addition to being a site of hormonal action, the mammary gland also secretes hormones. Relaxin is synthesised by the guinea-pig mammary gland, with the highest concentration being present during lactation (Peaker *et al.*, 1989) and this hormone may also be involved in mammary development, being essential for mammary growth in the pig (Hurley *et al.*, 1991). Goat mammary tissue secretes both oestradiol-17 β and prostaglandin F_{2 α} pre-partum, with the latter possibly being involved in the local mediation of lactogenesis stage II (Maule Walker, 1984).

1.7 PROLACTIN

If one hormone could be said to have a key role in the endocrine control of lactation it is prolactin. In addition to its effects on the mammary gland, prolactin has been attributed many biological actions and these can be grouped into five categories including growth, osmoregulation, reproduction, integumentary effects and synergism with steroids (Nicoll & Bern, 1972).

1.7.1 The gene family

Prolactin is one of a group of related peptide hormones within a multigene family, showing high homology at the gene level with both growth hormone and placental lactogen. Prolactins and growth hormones with distinct properties have been isolated from the pituitaries of all vertebrates, including fish, so the separation of these two peptides was an early step in evolution. From any one species there is marked sequence homology of growth hormone and prolactin, with about 25% of all amino acids being identical and the differing amino acids reflecting a high degree of

conservation (Moore *et al.*, 1982; Vonderhaar, 1987; Wallis, 1992).

There is also significant homology between the receptors for prolactin and growth hormone in areas of both extracellular and intracellular domains, suggesting a family of single membrane-spanning receptors. It has also recently been suggested that this family could be extended to include the receptors for erythropoietin, interleukins and others (Lesueur *et al.*, 1991).

1.7.2 Prolactin structure

Pituitary prolactin exists primarily as a non-glycosylated 20-24 kDa monomeric protein, although many other variants are also present (see Vonderhaar (1987)). The major form of ovine pituitary prolactin consists of 198 amino acids, with a molecular weight of 23 kDa (Li *et al.*, 1970) and a high glutamate and leucine content, which is a characteristic shared with many other prolactins (Kohmoto *et al.*, 1984). The members of the prolactin family also have two or three similarly positioned disulphide bonds, reflecting very similar secondary structures across species (Colosi *et al.*, 1982; Kohmoto *et al.*, 1984; Li *et al.*, 1970).

The structural similarity of prolactin, placental lactogen and growth hormone is reflected in overlapping activities in test systems. However, within a given species, each hormone will have a distinct biological role, defined by changing levels of the hormone in the circulation and by the quantity, affinity and specificity of receptors in the target tissues (Cowie *et al.*, 1980).

1.7.3 Prolactin secretion

Prolactin is synthesised and then released into the blood by mammatrophs present within the anterior pituitary gland. The mammatrophs are under dopamine-mediated inhibitory control by the hypothalamus, although additional prolactin inhibitory factors, including noradrenaline, have been proposed. Prolactin can control its own secretion, through both autocrine inhibition at the level of the mammatroph and stimulation of hypothalamic dopamine release. Thyrotropin-releasing hormone can act directly upon the pituitary to increase the release of prolactin, although its physiological role as a prolactin secretory factor is debated (Cowie *et al.*, 1980; Orstrom, 1990; Vonderhaar, 1987).

Prolactin is released from the anterior pituitary during lactation as a response to either suckling or milking. The prolactin release is a response to the tactile

stimulation of the teats and this can be modulated by sympathetic factors (Mena *et al.*, 1990a; Mena *et al.*, 1980). Following secretion, prolactin circulates in the blood apparently free of binding proteins, although there is recent evidence that these may be present in blood (Amit *et al.*, 1992) and milk (Postel-Vinay *et al.*, 1991). Plasma prolactin levels can be assessed by radioimmuno- or radio-receptor assay. However, the latter is a measure of hormone binding to the prolactin receptor and is not specific for prolactin alone, also detecting hormones such as placental lactogen which cross-react with the receptor (Cowie *et al.*, 1980).

Circulating prolactin levels are governed by season as well as by the stage of the oestrous cycle, pregnancy, lactation, stress, time of day, temperature and energy intake (Orstrom, 1990; Vonderhaar, 1987).

1.8 MECHANISM OF PROLACTIN ACTION

1.8.1 The Prolactin Receptor

It is generally accepted that to act at the cellular level, peptide hormones, such as prolactin, must first interact with receptors on the target cell surface membrane. A receptor is defined as a site which displays specific, saturable, high affinity binding for the hormone in question. The prolactin receptor is found in a variety of mammalian tissues, although most of the work on receptor characterisation has been done using the mammary gland or liver (Costlow, 1986).

1.8.2 Prolactin receptor structure

In addition to prolactin, prolactin receptors bind both placental lactogen and human growth hormone. Prolactin receptors are distinct from, but related to, growth hormone receptors (Boutin *et al.*, 1988) and are present in plasma membranes, Golgi, endoplasmic reticulum and lysosomal membranes, as well as in the soluble cytosolic fraction (Djiane *et al.*, 1987). The role of internal receptors in prolactin signal transduction is not known and only those receptors on the cell surface are exposed to the circulating hormone.

In rabbits, there is evidence that there are least two kinetically and structurally distinct forms of the prolactin receptor (isoreceptors) (Waters *et al.*, 1984). A prolactin receptor has been purified to near homogeneity from the rabbit mammary gland, giving a partially-sequenced protein of 42 kDa molecular weight (Waters *et al.*, 1990). Prolactin receptors of a similar size have also been purified from porcine

and bovine mammary tissue (Ashkenazi *et al.*, 1987; Berthon *et al.*, 1987). These receptors are similar in size to the "short-form" receptor (291 aa, 42 kDa) originally identified in rat liver by cDNA expression (Edery *et al.*, 1989). They are however, different to a "long-form" receptor (592 aa, 88 kDa), which can be identified in rat (Edery *et al.*, 1989) and rabbit (Murakami *et al.*, 1988; Sakai & Ike, 1987) mammary tissue and which, unlike the "short-form" receptor, is able to initiate milk protein gene transcription (Lesueur *et al.*, 1991).

Both "long" and "short" forms of the prolactin receptor consist of a single trans-membrane domain and are glycosylated. In the "short" form of receptor, glycosylation is involved in receptor dimerisation and modification of glycosylation does not affect receptor-ligand affinity (Rozakis-Adcock & Kelly, 1991). The cytoplasmic domain of the "short" form receptor is involved in receptor relocalisation, signal transduction, degradation and appears to influence ligand affinity (Rozakis-Adcock & Kelly, 1991).

The "long-form" receptor contains a long cytoplasmic region, although not all of this region is required for the transmission of the lactogenic signal (Ali *et al.*, 1992). It has been suggested that a stretch of 6 amino acids near the trans-membrane domain, possibly in concert with the long-form receptor's cytoplasmic tail, may play a critical role in transducing this signal (Ali *et al.*, 1992).

Perhaps surprisingly, in view of the "short-form" receptor's inability to initiate milk protein gene transcription (Lesueur *et al.*, 1991), this receptor type is the dominant form in lactating mammary tissue (Murakami *et al.*, 1988). Reproductive state has no significant effect on the relative proportions of the "long-" and "short-form" receptor mRNA types in the mammary gland, although the proportion of the "long-form" is slightly greater in this tissue than in the liver (Jahn *et al.*, 1991).

Other sized forms of prolactin binding subunit are also present in the rabbit mammary gland and appear to arise from differential splicing of a single receptor gene (Davis & Linzer, 1989; Dusanter-Fourt *et al.*, 1991), but are as yet uncharacterised.

1.8.3 Receptor - hormone complex formation

It is thought that each prolactin receptor interacts with one prolactin molecule (Ashkenazi *et al.*, 1987; Murakami *et al.*, 1988; Sakai & Ike, 1987). Although prolactin itself is not required for transduction of the hormone message (Djiane *et al.*,

1981b), subsequent dimerisation of the prolactin receptor may be necessary (Djiane *et al.*, 1987). In this respect, prolactin appears to differ from human growth hormone, which by crystallographic examination of the binding complex has been shown to bind two separate receptor molecules (Vos de *et al.*, 1992).

1.8.4 Prolactin signal transduction

Once prolactin is bound to its receptor, a signal is transmitted to the cell in order to elicit an appropriate biological response. For prolactin, the nature of this signal is still largely unknown and rather than working through a single second messenger, the ultimate biological response may be dictated by a series of events staged by other hormones and growth factors, all of which must act in concert (Vonderhaar, 1987). It is also possible that prolactin itself is directly responsible for mediating some of its actions, for internalised prolactin can be localised in the mammary cell nucleus (Nolin 1979; Seddiki & Ollivier-Bousquet, 1991).

As has already been discussed, it is the "long-form" prolactin receptor rather than the dominant "short-form" receptor which is able to initiate milk protein gene transcription (Lesueur *et al.*, 1991). From these separate actions, it would be expected that the two receptor forms operate through separate signal transduction pathways. However, it is also possible that changes in the relative amounts of the different receptor types and in the concentration of circulating prolactin, could influence the nature of the prolactin signal by affecting the relative proportions of the different dimerised receptors following binding of the hormone.

The Nb2 prolactin receptor, a mutant of the long-form prolactin receptor missing a large part of cytoplasmic domain, is fully capable of transducing the lactogenic signal. This and the mitogenic response, occurs in the absence of an ATP/GTP binding site (Ali *et al.*, 1992). However, a protein containing such a binding site may be recruited on receptor activation, for GTP inhibits lactogenic hormone binding to its receptor (Vonderhaar *et al.*, 1991). The Nb2 receptor has also been demonstrated to rapidly induce protein tyrosine phosphorylation (Rui *et al.*, 1992). This provides strong evidence for a tyrosine kinase present within the activated prolactin receptor complex being involved in prolactin signal transduction.

Rapid effects of prolactin on protein secretion have been reported in lactating rabbit mammary tissue fragments *in vitro* (Seddiki & Ollivier-Bousquet, 1991). In contrast, prolactin's effects on milk protein gene expression occur over a period of

many hours (Matusik & Rosen, 1978). These contrasting acute and chronic effects may reflect separate signalling pathways. A possible mechanism for prolactin's acute actions is suggested by the observation that prolactin induces a transient release of free arachadonic acid, released from membrane phospholipids by the action of phospholipase A₂ (Rillema *et al.*, 1986) and inhibition of subsequent prostaglandin synthesis can affect protein secretion (Blachier *et al.*, 1988; Seddiki & Ollivier-Bousquet, 1991). This effect may be a consequence of arachadonic acid metabolites activating protein kinase C (Shearman *et al.*, 1989), which has, in turn, been shown to stimulate Ca²⁺-independent exocytosis in permeabilised mammary cells (Turner *et al.*, 1992b). Activation of protein kinase C by prolactin has also been shown to occur in mammary gland explants (Caulfield & Bolander, 1986) with cellular translocation of protein kinase C also being affected in NOG-8 cells, a mouse mammary cell line (Vonderhaar *et al.*, 1991).

It has also been suggested that prolactin's mitogenic actions are mediated through *synlactin*, a prolactin-induced somatomedin-like molecule produced by the liver (Nicoll *et al.*, 1985). Prolactin may therefore act both directly and indirectly on target tissues.

1.9 PROLACTIN RECEPTOR REGULATION

1.9.1 Determination of prolactin binding

The regulation of the number of receptors present on the surface of a target cell is a complex phenomenon. Various physiological factors influence prolactin binding in the mammary gland and these act by modulating prolactin receptor number rather than affinity (Djiane *et al.*, 1977; Jones & Parker, 1983). Although the study of Perry and Jacobs (1978) claimed to demonstrate small increases in receptor affinity in late-lactation, this may simply have reflected an effect on the contribution of non-specific binding to the estimated receptor affinity as a consequence of changes in the level of specific prolactin binding (Chamness & McGuire, 1975). The modulation of the cell's sensitivity to circulating prolactin, resulting from alterations in hormone receptor number, is in addition to the control offered by changing plasma hormone concentrations.

Many studies have looked at the control of the prolactin receptor in the mammary gland and the majority have utilised the binding of radiolabelled hormone to tissue slices, microsomal membranes or enzymatically-dispersed whole cells

(Costlow, 1986). The masking of prolactin receptor sites by endogenously-bound hormone must be considered in studies where prolactin has been present. Occupied receptor sites may be stripped of the bound hormone by either acid washing of intact cells (Costlow, 1986) or 4 M-MgCl₂ treatment of membranes (Kelly *et al.*, 1979; Van der Gugten *et al.*, 1980), although partial purification of the membrane may result from the latter treatment as a consequence of a non-random loss of protein (Hayden & Smith, 1981). Recent studies have utilised monoclonal antibodies which bind to the prolactin receptor away from the occupied binding site (Jahn *et al.*, 1991). Some investigators have attempted to desaturate prolactin receptors *in vivo*, by administering bromocriptine to suppress circulating prolactin (Djiane *et al.*, 1987), although this will affect prolactin's regulation of its own receptor.

In both rodents and ruminants, the number of prolactin receptors in the mammary gland rises during gestation and this parallels changes in the levels of circulating prolactin and placental lactogen (Kazmer *et al.*, 1986; Kelly *et al.*, 1979). A further increase in mammary prolactin receptor content occurs during lactation and this is dependent upon both sustained prolactin release (Bohnet *et al.*, 1977) and continued milk removal (Hayden & Smith, 1981; Moore & Forsyth, 1980; Stewart, 1984). In mice, mammary prolactin receptor number is correlated to milk yield, increasing during early lactation, reaching a maximum in mid-lactation (day 10) before declining thereafter (Sakai *et al.*, 1985). These studies suggest that changes in mammary prolactin receptor number are of importance in regulating mammary function and development.

1.9.2 Receptor turnover

The prolactin receptor is a short-lived molecule, with a half-life of less than 3 hours (Djiane *et al.*, 1982; Kelly *et al.*, 1975). The mRNA for the prolactin receptor appears to have a long half-life, with rapid modification of receptor levels occurring at the translational or degradative level (Djiane *et al.*, 1982). It is probable that prolactin receptor expression is under both transcriptional and translational control, for alterations in hormone binding are not completely accounted for by receptor mRNA levels (Jolicoeur *et al.*, 1989). Receptors are subject to continuous turnover, with consequent continuous trafficking between cellular compartments as newly synthesised receptors replace those that are degraded. It is thought that Golgi membranes are the site of storage for receptors before their insertion on the cell

surface (Costlow, 1986). Once bound, it is believed that the prolactin hormone-receptor complex forms clusters in clathrin-coated pits, before internalisation from the cell surface (Scmid, 1992). Both the cell surface expression of receptors and the subsequent internalisation to intracellular sites, before lysosomal degradation, are energy-dependent (Costlow & Hample, 1982a).

1.9.3 Self-regulation of the prolactin receptor

Prolactin induces both up- and down- regulation of its own receptor. High circulating concentrations of prolactin result in the internalisation of receptors from the plasma membrane to the Golgi membranes before lysosome-dependent degradation (Djiane *et al.*, 1981b). This down-regulation of total receptor number is rapid and is maximal after 1 hour, before recovery over 24 hours (Djiane *et al.*, 1981b). Sustained increases in prolactin concentrations result in up-regulation of the receptor over a period of days (Kelly *et al.*, 1984).

1.9.4 Regulation of prolactin receptors by other hormones

In addition to self-regulation, the prolactin receptor is regulated by a variety of other hormones. Glucocorticoid is required in mammary cells for the maintenance of prolactin receptor number (Sakai & Banerjee, 1979) and acts to increase receptor number *in vitro* (Sakai *et al.*, 1979). Progesterone acts on the mammary gland to antagonise prolactin action (Djiane & Durand, 1977), by inhibiting prolactin receptor gene expression (Jahn *et al.*, 1991) and progesterone's inhibition of lactogenesis stage II is due, at least in part, to an effect on mammary prolactin receptor number. Oestrogen also acts to inhibit prolactin's action, through down-regulation of the prolactin receptor (Bohnet *et al.*, 1977), whilst the thyroid hormone T₃ up-regulates the mammary prolactin receptor, both *in vitro* and *in vivo*, and also affects the concentration of circulating prolactin (Bhattacharjee & Vonderhaar, 1984; Bhattacharya & Vonderhaar, 1979).

1.9.5 The membrane environment and cryptic receptors

Interpretation of many studies is complicated by changes in the levels of circulating prolactin, as well as possible effects on membrane fluidity resulting from prolactin action (Alhadi & Vonderhaar, 1982). The interaction of prolactin with its receptor results in the inclusion of prostaglandins into the plasma membrane, with

resultant changes in membrane lipid composition and fluidity (Dave, 1987). This can affect prolactin binding, possibly as a result of altering the availability of so-called "cryptic" receptors which are normally hidden within the plasma membrane (Dave & Knazek, 1980). In the tamar wallaby, the proportion of cryptic prolactin binding sites is reduced as lactation progresses (Nicholas, 1988). There is also a link between the effects of tamoxifen on prolactin binding and the response of mouse mammary cells to prolactin *in vitro* (Biswas & Vonderhaar, 1991), although this may simply reflect competition between these ligands for the prolactin receptor (Biswas & Vonderhaar, 1991). With the current state of knowledge, it is not known whether the changes in prolactin binding seen with membrane modification are of physiological significance in regulating prolactin action.

1.9.6 Prolactin transport into milk

A variety of immuno-detectable and bioactive hormones are known to be present in the milk of several species and this is probably true of all milks. Although implicated, there is no proven biological role in neonatal development for hormones in milk and their role in mammary function is unknown (Peaker & Neville, 1991).

The concentration of prolactin in milk, like that of many hormones, approximates and on some occasions greatly exceeds that found in the blood (Malven & Keenan, 1983). It is clear that prolactin arrives in the milk by transepithelial, as opposed to paracellular or intercellular, transfer. Prolactin is present within various portions of the mammary epithelial cell at concentrations greater than that found in milk (Malven & Keenan, 1983). The hormone is transported from the basal membrane of the cell to the apical regions before being discharged into the milk (Nolin 1979). Using immunocytochemical studies, a link was demonstrated between intracellular prolactin incorporation and the lactational activity of the cell (Nolin & Bogdanove, 1980). It is not known whether the hormone is associated with the receptor during this transfer process or what proportion of the hormone is ultimately transferred, for at least part is subject to degradation (Fleet *et al.*, 1992; Shiu, 1980).

1.10 GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTORS

Hormones other than prolactin are involved in mammary function and in recent years there has been much interest in the role of growth hormone in lactation. Growth hormone is the major galactopoietic hormone in ruminants (Cowie *et al.*,

1980) and exogenous administration produces marked increases in milk yield (Cowie *et al.*, 1980; Prosser & Mepham, 1988).

Growth hormone, or somatotrophin as it is also known, is secreted by somatotrophs within the anterior pituitary and is related to both prolactin and placental lactogen. The growth-promoting actions of growth hormone are brought about by secondary mediators, now identified as insulin-like growth factors (Daughaday *et al.*, 1987). Insulin-like growth factors are synthesised by most body tissues, with those in the circulation being released from the liver and being bound non-covalently to large molecular weight binding proteins, which may coordinate their action (see (Baxter, 1991)). Insulin-like growth factors are potent mitogens for many cells, including mammary cells and are also involved in cellular differentiation (Forsyth, 1989; Hill, 1989).

Although mRNA for the growth hormone receptor is present in rabbit and bovine mammary tissue (Glimm *et al.*, 1990; Jammes *et al.*, 1991) and growth hormone binding proteins are present in rabbit milk (Postel-Vinay *et al.*, 1991), the receptor itself has not yet been detected in the mammary gland (Akers, 1985; Kazmer *et al.*, 1986). There is no apparent direct effect of growth hormone on ruminant mammary tissue *in vitro* (Gertler *et al.*, 1983; Goodman *et al.*, 1983) and local arterial infusions of growth hormone do not affect milk yield (McDowell *et al.*, 1987). It is thought that growth hormone acts indirectly on the mammary gland, as a consequence of increased levels of circulating insulin-like growth factor-I (IGF-I), but not IGF-II (Davis *et al.*, 1987; Prosser *et al.*, 1989). There are specific receptors for IGF-I and IGF-II in the ovine (Winder & Forsyth, 1987) and bovine mammary gland (Campbell & Baumrucker, 1986), with specific uptake of IGF-I, but not IGF-II, across the gland into the milk (Prosser & Fleet, 1992; Prosser *et al.*, 1991). In addition, IGF-I, but not IGF-II, affects mammary gland growth and differentiation *in vitro* (Duclos *et al.*, 1989; Winder & Forsyth, 1986). Systemic administration of IGF-I alone does not produce a sustained increase in milk yield (Davis *et al.*, 1989), although there is a milk yield and mammary blood flow response to local infusions of IGF-I (Prosser *et al.*, 1990).

In prolactin-deficient rats, both circulating insulin-like growth factor-I and -II are growth hormone dependent but neither can mimic growth hormone's action on the mammary gland (Barber *et al.*, 1992; Fawcett *et al.*, 1991; Flint *et al.*, 1992). It is possible however that the effects of IGFs may be coordinated by binding proteins

(such as IGF-binding protein-3) (Flint *et al.*, 1992). The precise mechanism of growth hormone's action on the mammary gland currently remains unknown, with effects on the mammary gland independent of homeorhetic effects (Bauman & Currie, 1980) in non-mammary tissues.

1.11 THE LOCAL CONTROL OF MILK SECRETION

In addition to systemic control by circulating hormones, the rate of milk secretion by the mammary gland is modulated locally by changes in the frequency and completeness of milk removal.

1.11.1 Milking frequency studies

It was the increase in milk yield that resulted from the unilateral effect of frequent milking, in goats (Linzell & Peaker, 1971) and cows (Morag, 1973), that first suggested that the rate of milk secretion by the mammary gland is under local control. More frequent removal of milk from a gland produces an increased rate of milk secretion when hourly (Linzell & Peaker, 1971) or thrice daily milk removal (Henderson *et al.*, 1983) is compared to twice daily milking. The rate of milk secretion is increased only in the gland that receives the additional milkings and is limited to the daily period in which the extra milking is applied. These effects are rapidly reversed when the gland is returned to twice daily milking (Henderson *et al.*, 1983).

This unilateral effect on milk secretion is independent of systemic hormonal factors released in response to teat stimulation, because an effect can be obtained in autotransplanted (denervated) glands (Linzell & Peaker, 1971). The milk yield response is dependent on the removal of milk from the denervated gland, for massage of the gland without milk removal has no effect on milk secretion (Linzell & Peaker, 1971). The effect of frequent milking is not simply due to the relief of pressure from milk stored within the gland, for the replacement of removed milk with an inert sucrose solution does not prevent the increased rate of milk secretion which results from the additional milking (Fleet & Peaker, 1978; Henderson & Peaker, 1984). In fact, in the goat, rising intra-mammary pressure does not affect the rate of milk secretion until after 24 hours of milk accumulation (Fleet & Peaker, 1978).

1.11.2 A chemical factor

Linzell and Peaker (1971) suggested that the stimulation of milk secretion with hourly milking was a result of the more frequent removal of a milk constituent. This chemical would negatively feedback to the secretory cell to limit milk secretion. Dilution of the milk stored in the udder with an inert isosmotic solution produces an increased rate of milk secretion, an effect which is compatible with the reduction in concentration of a chemical inhibitor in the milk (Fleet & Peaker, 1978; Henderson & Peaker, 1987). Evidence for the local control of milk secretion has also been obtained in the human, with it being demonstrated that as successive feeds by the infant empty the breast of milk, the rate of milk synthesis by the gland increases four-fold (Daly *et al.*, 1992).

The apparent ability of the milk secretory cells to produce a factor, the feedback inhibitor of lactation (FIL) (Addey *et al.*, 1991b), which acts to inhibit their own activity has been termed the autocrine control of milk secretion (Peaker & Wilde, 1988). Several metabolic processes other than milk secretion are regulated by apparent autocrine mechanisms. The term "autocrine" control was first used to describe the ability of tumour cells to regulate their own proliferation.

1.11.3 Isolation of the chemical factor

Wilde and co-workers (1987a) identified a fraction of goat whey proteins of 10,000-30,000 Da molecular weight, which inhibited lactose and casein synthesis in rabbit mammary tissue explants. This inhibition was rapid, dose-dependent and reversible. The heat-labile inhibitory fraction was active *in vivo*, inhibiting milk accumulation in both lactating rabbits (Wilde *et al.*, 1987a; 1988b) and goats (Wilde *et al.*, 1988a) when introduced into the mammary gland. Other milk fractions had no significant effect. The results from these experiments were compatible with the chemical inhibitor hypothesis put forward by Linzell and Peaker (1971).

The 10-30 kDa whey fraction from both human milk (Prentice *et al.*, 1988) and dry goat secretion (Blatchford *et al.*, 1985) demonstrates a similar inhibition of lactose and casein secretion in the rabbit mammary explant bioassay. The active constituent of the 10-30 kDa whey fraction (FIL) has been purified from caprine (Addey *et al.*, 1991b), bovine (Addey *et al.*, 1991a) and human milk (Dr. C.J. Wilde, unpublished work). The properties of caprine FIL will be discussed in detail elsewhere.

1.11.4 The site of inhibitor action

During lactation, milk is secreted into the secretory alveoli. In dairy animals, milk can be also stored in the capacious large ducts and cistern, with the distribution of milk between the secretory and storage compartments changing as milk accumulates within the gland (Peaker & Blatchford, 1988). Milk stored in the cistern does not exert an effect in terms of the inhibition of milk secretion (Henderson & Peaker, 1987).

There is considerable variation between dairy animals in the relative proportions of the alveolar and cisternal milk storage fractions and this can affect the efficiency of milk secretion. A goat possessing a relatively small alveolar volume has a relatively high rate of milk secretion per unit mammary gland volume, by virtue of storing a greater proportion of milk of its milk at a site where FIL is inactive (Peaker & Blatchford, 1988). In addition, cows with proportionately large cisterns are more tolerant of once daily milking (Knight & Dewhurst, 1992) and less responsive to thrice daily milking (Dewhurst & Knight, 1992). This work demonstrates that it is the milk stored within the secretory alveoli, and therefore in contact with the secretory cell apical surface, that inhibits milk secretion.

1.11.5 Extended changes in milking frequency or efficiency

Short term changes in milking frequency increase milk yield by approximately 10% (eg an 8% increase being reported following 7 days of unilateral thrice daily milking (Henderson *et al.*, 1983)). Even larger increases result from sustained frequent milking (Henderson *et al.*, 1985). Thrice-daily milking of one udder half for 9 months produces a total milk yield that is 30% higher than that from the twice-daily milked gland, with milk yield of the thrice-daily milked gland being 43% higher than the twice-daily milked gland at week 40 of lactation (Henderson *et al.*, 1985).

Mammary epithelial cell differentiation is stimulated after 10 days of unilateral thrice-daily milking, with cell growth after 37 weeks of continued treatment (Wilde *et al.*, 1987b). This is compatible with the 37% greater gland weight for the thrice-milked gland than the twice-milked gland, observed by Henderson *et al.* (1985) after 36-38 weeks of thrice daily milking. Similar effects on cell differentiation were obtained in dairy cows following 4 weeks of four-times daily milking of diagonally opposed glands (Hillerton *et al.*, 1990).

In contrast to the effects of more frequent milking, cellular differentiation is

reduced by incomplete milk removal. Incomplete milking of one gland for a long (24 week) period in the goat, reduced the weekly milk yield by 23.8% when compared to the twice-daily milked gland. Significantly lower enzyme activities in the incompletely milked glands were coupled with reduced rates of milk protein synthesis in freshly-prepared explants. This is indicative of cellular de-differentiation (Wilde *et al.*, 1989b).

In summary, changes in the frequency, or efficiency, of milk removal affect the rate of milk secretion within hours. Over a period of weeks to months of frequent milking, the mammary cell population undergoes increased cellular differentiation, producing an increased yield per cell. Subsequently, an extended period of frequent milking stimulates growth of the treated gland, with an increased milk yield as a consequence of a larger secretory cell population.

1.11.6 Local modulation of hormonal sensitivity

Changes in mammary differentiation are associated with changes in hormone receptor number. In the goat, one consequence of more frequent milking is an increase in mammary secretory cell prolactin receptor number. Conversely, a reduction in number results from incomplete milking (McKinnon *et al.*, 1988). A greater response to frequent milking in hypoprolactinaemic animals also suggests that the hormonal sensitivity of the mammary gland is modulated by milk removal (Knight *et al.*, 1990a). This effect is apparently specific for prolactin, inasmuch as there was no effect of milking frequency on IGF-I receptor number or affinity (Wilde *et al.*, 1990).

There is also strong evidence for local control of milk secretion in the tamar wallaby and this appears to be mediated, at least in part, through changes in mammary prolactin receptor number. In this species, mammary glands develop and involute independently of each other as they individually support young at different stages of development (Tyndale-Biscoe *et al.*, 1984). This development is dependent upon suckling and the changes in suckling pattern that result from the offspring becoming mobile (Nicholas, 1988). Following parturition, the newborn offspring attaches to and suckles from a single mammary gland and the development of this gland is matched, as lactation proceeds, by an increasing number of mammary prolactin receptors (Bird *et al.*, 1992). Concurrently, the non-suckled glands regress and this is also matched by a decrease in mammary prolactin binding (Stewart, 1984).

Therefore, the changes in mammary development are possibly mediated through changes in mammary prolactin receptor number, for the hormonal environment is similar for both developing and regressing glands.

Further evidence for a relationship between mammary prolactin receptor number and differentiation is provided by studies in the rat. Unilateral ligation or removal of mammary gland teats reduces prolactin binding during pregnancy, with this becoming significant during early lactation (Moore & Forsyth, 1980). In addition, unilateral ligation during lactation itself produces milk stasis in the sealed glands within 12 hours and this is accompanied by a reduction in free prolactin receptor number (Hayden & Smith, 1981).

1.11.7 Autocrine - endocrine interactions

As described above, there are several phases in the mammary gland's response to changes in the frequency or completeness of milking and these are accompanied by changes in hormone receptor number. There is evidence that the local effects on cell differentiation may be exerted by the same autocrine inhibitor which is responsible for the acute local control of milk secretion. The 10-30 kDa goat whey fraction reversed mammary differentiation in lactating rabbit mammary glands *in vivo* (Wilde *et al.*, 1988b) and inhibited hormone-dependent induction of casein synthesis and fatty acid synthetase activity in mouse mammary epithelial cells *in vitro* (Wilde *et al.*, 1991). Furthermore, this whey fraction stimulated intracellular casein degradation in freshly-prepared goat mammary explants, a process which appears to be inversely related to the degree of secretory cell differentiation (Stewart *et al.*, 1988; Wilde *et al.*, 1989a). Though mammary prolactin receptor number was not measured in these studies, effects on mammary differentiation are possibly mediated through alterations in mammary prolactin receptor number, which would then modulate the gland's responsiveness to circulating hormone.

1.11.8 Systemic and autocrine interactions

A milk yield response to more frequent unilateral milking is dependent upon the nutritional status of the animal. Underfed goats which are in negative energy balance suffer a reduction in total milk yield and are not responsive to hourly milking (Blatchford & Peaker, 1982; Henderson *et al.*, 1983). In these circumstances, the rate of milk secretion is directly limited by the nutrient supply to the mammary gland and

the relief of local inhibition cannot be supported metabolically by the animal.

Treatment of ruminants with exogenous growth hormone results in an increased milk yield as a consequence of homeorhetic repartitioning of nutrients towards the udder. Exogenous growth hormone can be thought of as having a systemic effect upon milk secretion (Bauman *et al.*, 1985). Goats treated with bovine growth hormone respond to the effects of increased milking frequency, with an additive increase in milk yield of up to 55% (Knight *et al.*, 1990b), with similar additive responses also being seen in dairy cows (Knight, 1992) and sheep (Pell *et al.*, 1989). Treatment with bovine growth hormone accelerates, or augments, the differentiative response of the gland to frequent milking and stimulates additional growth of the gland through mammary cell hypertrophy in both goats and cows (Knight *et al.*, 1990b; Knight *et al.*, 1992). In goats, the response of lactational persistency differs from that of absolute milk yield, in that a synergistic improvement in persistency is seen only with the combined treatments of frequent milking and bovine growth hormone (Knight *et al.*, 1990b). There is no effect of either treatment alone on lactational persistency (Bauman *et al.*, 1985; Henderson *et al.*, 1985) and neither regime amplifies the gland's response to the other treatment (Knight *et al.*, 1992). This evidence points to separate mechanisms of action for the autocrine inhibitor of milk secretion and exogenous growth hormone.

1.12 CHANGES IN SUCKLING INTENSITY

Local changes in milk secretion and mammary differentiation, resulting from unilateral changes in milking frequency, are independent of the systemic effects arising from the milking stimulus. For an animal suckling its young, alterations in suckling intensity resulting from litter growth, weaning or a reduction in number may affect mammary development. In rats, a reduction in litter size at mid-lactation does not alter pup live weight gain, but does cause partial mammary regression. Systemic factors could be responsible, maternal prolactin concentrations are depressed and both insulin and food consumption levels lowered as a result of the reduced suckling intensity (Grigor *et al.*, 1984). An increase in pup number raised both maternal food consumption and insulin and prolactin levels, as well as increasing mammary differentiation. In rabbits, the transient reduction in milk yield from an individual suckled gland arising as a result of a reduced suckling intensity, could be reversed by administration of exogenous ovine prolactin (Mena *et al.*, 1974).

1.13 CESSATION OF LACTATION

When milk removal ceases, milk accumulation will continue until secretion is arrested. Factors other than local feedback by the autocrine inhibitor, such as mammary distension (Fleet & Peaker, 1978), are likely to be involved in the final cessation of lactation. In dairy cows and goats, milking is usually stopped when the milk yield is relatively low, but laboratory animals are often weaned when the females are still yielding appreciable quantities of milk. This type of cessation of lactation has to be contrasted with self-weaning when the young continue to be suckled until the mammary glands are producing only very little milk (see (Lascelles & Lee, 1978)).

It is important to distinguish between the systemic factors resulting from the removal of the suckling stimulus and the local effects resulting from milk accumulation within the mammary gland. In the rat, the arrest of milk secretion following complete removal of the litter can be attributed to the loss of the suckling stimulus and hormonal support (Hanwell & Linzell, 1973). In the non-suckled rat, milk secretion is unimpeded for the first 4 hours of milk accumulation and then declines between 4 and 8 hours (Hanwell & Linzell, 1973). The decline in milk accumulation is accompanied by reductions in both mammary blood flow and cardiac output, which can be restored by suckling (Hanwell & Linzell, 1973) or administration of either ovine prolactin or growth hormone (Hanwell & Linzell, 1972).

In "teat-sealed" suckled rats, milk accumulation itself is responsible for the halting of milk secretion, since the endocrine response to suckling is maintained. In sealed glands, milk accumulation continues for 8 hours without an effect on blood flow (Hanwell & Linzell, 1973). There is a decline in metabolic activity within 16-24 hours (Jones, 1968), coupled to a reduction in amino acid uptake (Vina *et al.*, 1981) and fatty acid synthesis (Levy, 1964); these changes being accompanied by a reduction in prolactin binding (Hayden & Smith, 1981). Finally, blood flow decreases when capillary closure occurs after 36-48 hours (Silver, 1956).

In goats, mammary distension is responsible, at least in part, for the cessation of lactation. The continued replacement of milk in one gland, with an inert sucrose solution, resulted in the cessation of milk secretion after 24-48 hours (Fleet & Peaker, 1978). The arrest of secretion is not due to a primary effect of mammary distension on blood flow resulting from occlusion of the capillaries (Peaker, 1980), although

reduced flow possibly occurs in the long term (Silver, 1956) as a result of a reduced production of vasodilators. The cessation of secretion appears to be a result of either an increased back pressure across the apical membrane opposing osmotic water movement into milk, or an effect of stretching on cell structure leading to a loss of secretory activity (Peaker, 1980). Localised changes in milk composition occur in the un milked gland, in both goats (Fleet & Peaker, 1978) and cows (Wheelock *et al.*, 1967) and these changes are compatible with the local disruption of the mammary epithelium as milk accumulates. However, the distension resulting from the milk accumulation alone is not sufficient to rupture the mammary epithelium (Peaker, 1980). The alternative mechanisms for the epithelial disruption are either a failure of the inactive secretory cells to maintain the structure of the junctional complexes, or the accumulation of chemical factors in the stored milk which affect permeability (Peaker, 1980). For a review on mammary involution, see Hurley (1989).

1.14 MAMMARY FACTORS

In addition to the autocrine inhibitor of milk secretion (Wilde & Peaker, 1990), other factors are produced by the mammary gland which are involved in controlling mammary development. The idea that these factors act *in vivo* via autocrine, paracrine or even endocrine mechanisms has gained popularity. Such factors have possible stimulatory or inhibitory actions on mammary growth and include insulin-like growth factors (Winder & Forsyth, 1986), epidermal growth factor (Taketani & Oka, 1983), transforming growth factors (Silberstein & Daniel, 1987) and mammary derived growth inhibitor (Kurtz *et al.*, 1990). Although not understood, it is thought that the local mammary factors modify the hormonal growth-promoting action on the mammary epithelium (Dembinski & Shiu, 1987), although as already described, it has also been suggested that IGF-I has local effects on milk yield (Prosser *et al.*, 1990). In addition, it has been proposed recently that transforming growth factor- β may have a role during pregnancy in suppressing casein accumulation (Robinson *et al.*, 1993). The reader is directed to a review by Forsyth (1989).

1.15 AIM OF THIS STUDY

The aim of this study was to gain insight into the autocrine mechanisms modulating endocrine regulation of the lactating mammary gland. It was hoped that this would provide an understanding of the local mechanisms by which milk removal regulates milk yield and secretory cell differentiation.

The initial objective was to determine whether the hormonal sensitivity of the mammary gland was affected by accumulation of milk within the gland (Chapter 3). Once an effect of milk accumulation on hormone receptor number had been demonstrated, I then pursued further studies aimed at determining if the effect was exerted by the same chemical mechanism that regulates milk secretion acutely, i.e. whether the feedback inhibitor of lactation (FIL) was responsible for these changes (Chapter 4).

Lactating goats were used in a preliminary study to investigate the relationship of mammary prolactin receptor number to the changes in milk yield resulting from alterations in milking frequency (Chapter 5).

The final objective was to determine the direct effects of FIL-containing milk fractions on prolactin receptor distribution and number in lactating mammary cells *in vitro* (Chapter 6). It was hoped that this would explain the apparent independence of milk yield and prolactin receptor number observed in Chapter 5, whilst investigating whether changes in mammary hormonal sensitivity mediate the acute effects of FIL on milk secretion.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

General laboratory chemicals were supplied by either Sigma Chemical Co., Poole, UK; BDH., Poole, UK or Boehringer Corp. Ltd., Lewes, UK unless stated otherwise. Bovine serum albumin (fraction V, 0.8% H₂O) was from Sigma Chemical Co., Poole, UK.

Cell culture media were purchased from either Gibco Ltd., Paisley, UK or Northumbria Biologicals Ltd., Cramlington, UK.

The ovine prolactin (oPrl, NIDDK-o-Prl-19) used for iodination and in prolactin-binding studies was a gift from National Hormone and Pituitary Programme, Maryland, Baltimore, USA. Recombinant human insulin-like growth factor-1 (IGF-1) was a gift from Ciba-Geigy, Friebourg, Switzerland.

All water was single-distilled mains water.

2.1.2 Radiochemicals

Radiochemicals were from Amersham International, Amersham, UK or ICN Flow, Irvine, UK. The ¹²⁵I- activity of samples was determined using either a Cobra auto-gamma counter (74% efficiency, Canberra Packard, Pangbourne, UK) or a model 1270 Rackgamma II (75% efficiency, LKB., Croydon, UK). ¹⁴C- and ³H-activities were determined using a 1600TR liquid scintillation analyser with "emulsifier safe" scintillation fluid (both from Canberra Packard).

2.1.3 Animals

Mice and rabbits were housed individually in an animal house under a 12 hour light-dark cycle (light from 08.00 - 20.00h). The day of parturition was taken as day 0 of lactation. Mice (Tucks No 1 strain, A. Tuck and Son, Essex, UK) had diet CRMX (SDS, Manea, Cambridgeshire, UK) and tap-water available *ad libitum*. Primiparous Dutch-breed rabbits were obtained during mid-pregnancy from either Shrubacre Rabbits, Suffolk, UK or Hyline Rabbits, Cheshire, UK. Both diet CRB (SDS) and tap-water were available *ad libitum*.

British Saanen goats were from the Institute herd and were routinely milked twice daily at approximately 08.00 and 16.00 h. They were fed 1.5 - 1.8 kg concentrates (16.5% CP, 12.5 MJ/kg ME; Goat Mix No. 1, Edinburgh School of Agriculture, Edinburgh, UK) daily, receiving one-half of the ration at each milking, with hay and water available *ad libitum*.

2.2 IODINATION OF PEPTIDES

^{125}I was incorporated into protein using the Iodogen[™] iodination reagent (Pierce Europe BV, Oud-Beijerland, Netherlands) first described by Fraker and Speck (1978). Microtubes (1.5 ml) were coated with 30 μl of Iodogen (0.05 mg/ml in chloroform), evaporated to dryness at room temperature and stored at -20°C until required. Carrier-free ^{125}I (5-10 μl , approximately 0.5 mCi) was incorporated into the peptide (10 μl of 500 $\mu\text{g}/\text{ml}$ in 7.5% NaHCO_3) in an Iodogen-coated microtube in the presence of 10 μl of 0.5 M- PO_4 buffer (pH 7.4). After 15 minutes, the mixture was transferred with 200 μl of KI (2% w/v) to a minicolumn. This consisted of a 5 ml plastic syringe-barrel containing 3 g of Sephadex G-10 (Pharmacia, Uppsala, Sweden) equilibrated with radioimmunoassay buffer (0.05 M- NaH_2PO_4 at pH 7.4 containing 0.25 M- NaCl , bovine serum albumin (0.5% w/v, radioimmunoassay grade) and 7.7 mM- NaN_3). Fractions were collected from the minicolumn every 60 s (approximately 0.5 ml) and ^{125}I - activity determined using a Geiger counter (Mini-Instruments Ltd., Essex, UK), until the second peak of radioactivity containing unincorporated ^{125}I had been obtained.

Incorporation of ^{125}I into the peptide was verified by trichloroacetic acid (500 μl , 10% w/v) precipitation of a 50 μl protein fraction of known radioactivity. The precipitate was recovered by centrifugation (30 s, Eppendorf, Netheler + Heinz, GmbH.) after 10 minutes at room temperature and counted for ^{125}I activity. In a successful iodination, more than 95% of ^{125}I in the major protein fraction was trichloroacetic acid precipitable.

Radioimmunoassay buffer (1-2 ml) was added to the major protein fraction and aliquots stored for up to two months at -20°C .

The specific activity (A_0) of the iodinated peptide was calculated according to the following equation :-

$$\text{Specific Activity } (\mu\text{Ci}/\mu\text{g}) = (B+D)/(B+D+E) \times (A/1000) \times (500/C)$$

where A = ^{125}I added (cps at 60cm),

B = residual ^{125}I not transferred to minicolumn
(cps at 40cm)

C = quantity of protein iodinated (μg),

D = ^{125}I present in total protein peak (cps at 40cm),

E = ^{125}I present in iodine peak (cps at 40cm),

The specific activity was corrected on every occasion that the iodinated peptide was used, in accordance with the following equation:-

$$\text{Corrected specific activity } (\mu\text{Ci}/\mu\text{g}) = A_0 \times e^{(-\ln 2 \times t / t_{1/2})}$$

where A_0 = original specific activity ($\mu\text{Ci}/\mu\text{g}$),

e^{\wedge} = natural antilogarithm,

$\ln 2$ = natural logarithm of 2,

t = days since iodination,

$t_{1/2}$ = half life of ^{125}I (60 days).

2.3 PREPARATION OF GOAT MILK FRACTIONS

2.3.1 10-30 kDa Goat Whey Fraction

The goat milk fraction, containing whey proteins in the approximate range of 10,000-30,000 Da, was prepared essentially as described by Wilde *et al* (1987a). One litre of whole goat's milk was collected at the morning milking and protease inhibitors (2 g of ϵ -amino n-caproic acid, 0.348 g phenylmethylsulphonyl fluoride in 10 ml ethanol) were added before defatting by centrifugation (2200 g_{av} , 10 mins, 10°C) and filtration through glass wool. The defatted milk was centrifuged (30,000 g_{av} , 120 mins, 10°C) and the clear supernatant, which constitutes the whey fraction, filtered through a 0.2 μm pore-sized filter (Whatman International, Maidstone, UK). This was then subjected to ultrafiltration using filters with a nominal molecular weight cut-off of 30,000 Da (Minitan filtration system; Millipore Systems, Bedford, Mass., USA). The filtrate was dialysed against water for 24 hours at 4°C using tubing with a nominal molecular weight cut-off of 6000- 8000 Da (Spectrapor; Spectrum Medical Industries Inc., Los Angeles, USA), lyophilised and stored at -20°C.

2.3.2 Isolation of the feedback inhibitor of lactation (FIL)

The 10-30 kDa fraction of caprine whey proteins was resolved by anion exchange chromatography using a Mono Q HR 10/10 column (FPLC System, Pharmacia, Uppsala, Sweden), 10 mM-bis-tris propane (pH 7.0) and a 0 - 1.0 M-sodium acetate gradient. FIL was collected as the third resolved fraction (see Figure 4.1), (Addey *et al.*, 1991b). The protein fraction was dialysed against water for 24 hours at 4°C, lyophilised and stored at -20°C.

2.4 DNA ASSAY

The DNA content of samples was assessed by a fluorometric procedure (Labarca & Paigen, 1980). The DNA standard (calf thymus) was prepared in water at 1 mg/ml and stored at 4°C, with dilution to 20 µg/ml with assay buffer (0.1 M-NaH₂PO₄ at pH 7.4 containing 2 M-NaCl) before use. Fluorescent reagent was prepared by dissolving bisbenzimidazole (Fluka, Glossop, Derbyshire, UK) in water at 1 mg/ml and diluted to 2 µg/ml with assay buffer before use.

Samples were sonicated (15 s at setting 30; Kontes micro-ultrasonic cell disruptor, Buckard Scientific, Middlesex, UK) in assay buffer and an appropriate volume removed for assay. The DNA standard was used in the range of 0 - 200 µg and all assay volumes were made up to 2 ml with assay buffer before addition of 2 ml of fluorescent reagent. After 15 - 30 minutes at room temperature, fluorescence in the samples was measured on a model LS-5 luminescence spectrometer (Perkin Elmer Ltd., Beaconsfield, Bucks., UK) with an excitation wavelength of 356 nm, an emission wavelength of 445 nm and both input and output settings at level 5.

2.5 PROTEIN ASSAY

The protein content of samples was assessed as described by Bradford (1976), using bovine serum albumin as the protein standard (0.1 mg/ml). Dye-binding reactions took place in microtitration plates, with standard (0-8 µg) and assay wells being made up to 100 µl before the addition of 240 µl of fourfold diluted Bradford reagent (Bio-rad laboratories Ltd., Herts., UK). Coloured complex formation was assessed at a wavelength of 620 nm using a "Titertek multiskan" plate reader (Labsystems Ltd., Basingstoke, Hants, UK).

2.6 PREPARATION OF MICROSOMAL MEMBRANES FROM LACTATING MAMMARY TISSUE

All procedures were performed on ice, or at 4°C. Frozen mammary tissue (approximately 10 - 15 g) was thawed, weighed and chopped finely with scissors in 2.5 volumes (w/v) of membrane extraction medium (20 mM-Tris at pH 7.4 containing 0.3 M-sucrose and 1 mM-EDTA) supplemented with phenylmethylsulphonyl fluoride (0.2% v/v of 94 mg/ml in ethanol) and aprotinin (2% v/v of 0.83 mg/ml). The minced tissue was homogenised for 3x 20 seconds at 20,000 rpm, using a 20N head on a Polytron homogeniser (Kinematica, GmbH, Littau). Following centrifugation (2200 g_{av} , 10 min), the pellet was discarded and a sample of the supernatant was taken for subsequent determination of DNA content, before further centrifugation (15,600 g_{av} , 25 min). The supernatant from this step was centrifuged (98,900 g_{av} , 60 min) and the resultant microsomal membrane pellet was homogenised in 2 ml of membrane dilution buffer (20 mM-Tris pH 7.4 containing 10 mM- $CaCl_2$) supplemented with aprotinin (2% v/v as above) using a glass-glass homogeniser (Jencons Ltd., Leighton Buzzard, UK).

The pellet homogenate was diluted with 50 ml of membrane dilution buffer and collected by centrifugation (2200 g_{av} , 30 min), with this washing procedure being repeated following re-suspension of the pellet. The final pellet was re-suspended in 1 ml of membrane dilution buffer and a 200 μ l sample of this homogenate set aside. The remaining membrane homogenate was mixed with 4 ml of 4.5 M- $MgCl_2$, left for 10 minutes, diluted to 40 ml with membrane dilution buffer and collected by centrifugation (29,900 g_{av} , 40 min). Following re-suspension in membrane dilution buffer, the membrane protein concentration of both 4.5 M- $MgCl_2$ treated and non-treated samples was determined by Bradford (1976) protein assay, and samples stored at -20°C for subsequent determination of prolactin receptor content by radio-receptor assay.

2.6.1 Preparation of microsomal membranes from rabbit liver

Microsomal membranes were prepared from mid-pregnant (day 17) rabbit liver as described for mammary tissue, with the exception that the prepared membranes were not treated with 4.5 M- $MgCl_2$.

2.7 DETERMINATION OF PROLACTIN BINDING TO MICROSOMAL MEMBRANES (Radio-receptor assay)

Prolactin receptor number and affinity in microsomal membranes was estimated using the method of Hayden *et al* (1979).

The reactants in the [¹²⁵I]-oPrl binding assay were added to polypropylene microtubes in the following order:

(1) 100 μ l of microsomal membrane (0.9 mg/ml) in membrane dilution buffer (20 mM-Tris at pH 7.4 containing 10mM-CaCl₂),

(2) 100 μ l of unlabelled oPrl (0 - 4.35 μ M) in hormone diluent buffer (20 mM-Tris at pH 7.6 containing bovine serum albumin (1% w/v)) and

(3) 100 μ l of [¹²⁵I]-oPrl (0.5 x 10⁶ cpm/ml; 216 pM) in hormone diluent buffer.

The tube contents were vortex-mixed and incubated overnight at room temperature. The reaction was terminated by the addition of 0.5 ml of ice-cold saline (0.9% w/v) and centrifugation (2200 g_{av}, 30 minutes, 4°C), with the supernatants then being decanted. The ¹²⁵I activity of the pellets was determined and the specific prolactin binding calculated by subtracting non-specific binding of [¹²⁵I]-oPrl (binding determined in the presence of excess unlabelled oPrl) from total binding (in the absence of unlabelled oPrl).

2.7.1 Determination of IGF-I binding to mammary microsomal membranes

The radio-receptor assay for IGF-I was performed as described for oPrl, with [¹²⁵I]-IGF-I (50,000 dpm; 126 pM) being incubated, in triplicate, with 90 μ g of 4.5 M-MgCl₂ stripped mammary membrane. Specific binding of [¹²⁵I]-IGF-I was determined by subtracting the amount of radioactivity bound non-specifically in the presence of excess unlabelled IGF-I (222 nM).

2.8 ISOLATION OF LACTATING MOUSE MAMMARY CELLS

Mammary cells were isolated from lactating (day 10 \pm 1 of lactation) mice, suckling litters of 10 pups (adjusted at parturition). The enzymic dissociation procedure was modified from that described for pregnant mice by Wilde *et al* (1991). The culture of the isolated cells formed the basis of measurements of prolactin receptor subcellular localisation. In addition, the morphological and metabolic characteristics of the cells, including milk product synthesis and secretion, was determined.

Ingredient	Concentration in medium for:			
	Digestion	Wash	Incubation	Binding
buffer	Hanks	Hanks	Medium 199 ¹	Hanks
amino acids ²	normal strength	normal strength	normal strength	normal strength
D-glucose	1 mg/ml	1 mg/ml	1 mg/ml	-
bovine serum albumin	40 mg/ml	-	10 mg/ml	10 mg/ml
Hepes	-	-	3.2 mg/ml	3.2 mg/ml
NaHCO ₃	-	-	2.25 mg/ml	-
insulin	5 µg/ml	5 µg/ml	25 ng/ml	25 ng/ml
cortisol	10 ng/ml	10 ng/ml	10 ng/ml	10 ng/ml
bestatin	-	-	-	1.6 µg/ml
leupeptin	-	-	-	10 ng/ml
KCN	-	-	-	326 µg/ml
collagenase	7250 u	-	-	-
DNase I	-	30 µg/ml	-	-

¹ with Earle's salts

² minimum essential amino acids (medium Eagle)

Table 2.1. Recipes of media used for the isolation and incubation of, and determination of prolactin binding to, lactating mouse mammary cells.

2.8.1 Reagents:- Cell culture media

The details of the culture media used in experiments involving isolated lactating mouse mammary cells are given in Table 2.1. Media were made up at pH 7.4, sterilised through a 0.2 μ m pore filter (Gelman Sciences, Southampton, UK) and stored at 4°C until required. Hormones, protease inhibitors (bestatin and leupeptin), KCN and milk fractions, if included, were added to the culture media on the day before use. Collagenase (type 3, Worthington Biomedical, New Jersey, USA) and deoxyribonuclease (DNase I, Boehringer Group Ltd., Lewes, UK) were added to media on the day of use.

2.8.2 Preparation of chemicals

Reagents present in culture media were prepared in the following manner.

Hormones

Insulin (0.1 mg/ml) was prepared by dissolving 2 mg of insulin (from bovine pancreas, 24.4 I.U./mg) in 100 μ l of 0.34 M-NaOH before dilution with water.

Cortisol (0.1 mg/ml) was prepared by dissolving 2 mg cortisol (hydrocortisone-21-acetate) in 1 ml ethanol and diluting to 20 ml with water. This stock solution was stored for up to 4 weeks at 4°C, before subsequent dilution with water to 100 ng/ml.

Prolactin (from sheep pituitary, 32 I.U./mg) was prepared by dissolving 1 mg of oPrl in 250 μ l of 10 mM-Hepes (pH 8) before dilution with water to 100x the final concentration in the incubation medium.

Bestatin and leupeptin

Bestatin and leupeptin were dissolved in 0.9% (w/v) NaCl at 1 mg/ml and 0.1 mg/ml respectively and stored separately in aliquots at -20°C.

Digitonin

A stock solution (5% w/v) was prepared by dissolving 500 mg digitonin (Boehringer Group Ltd., Lewes, UK) in 10 ml of 1 mM-Tris (pH 7.4). This solution was brought to the boil over a flame, cooled, filtered (0.2 μ m) and the volume returned to 10 ml. Storage was at 4°C, with the digitonin being redissolved by warming in hot water (Mabon, 1990).

Saponin

A solution was prepared in binding medium as required.

Potassium Cyanide (KCN)

This was weighed by difference, in a fume-hood, into a sealable tube and dissolved in water at 100 mM. Cyanide-containing solutions were neutralised with sodium hypochlorite (14% w/v available chlorine) before disposal with excess water.

Milk fractions

For experiments involving isolated cells, milk fractions were made up at 4x relative to their milk concentration in 10 mM-Hepes (pH 7.4) and 0.2 μm filter-sterilised before addition to culture medium.

2.8.3 Preparation of lactating mouse mammary cells

Digestion

Approximately 4 g of mammary tissue was collected from a single mouse, which had been killed by cervical dislocation and drenched in 70% (v/v) ethanol. Excess milk was removed by washing in 20 ml of Hanks Buffer (pH 7.4) and the tissue was chopped finely using curved scissors. Digestion was performed in 40 ml of digestion medium, using an orbital incubator (Gallenkamp, Loughbrough, UK) at 37°C and 120 rpm with agitation by pipette every 15 minutes.

After approximately 100 minutes, when the cells were predominantly in clumps of 10-20 cells, the digest mixture was filtered through a 150 μm pore nylon mesh (Henry Simon Ltd., Stockport, UK) held in a 45 mm filter support (Nalgene, Rochester, New York, USA). The cells were harvested by gentle centrifugation (80 g_{av} , 4 min, room temperature), resuspended in 20 ml of wash medium and re-harvested. This wash step was repeated three more times. The final cell pellet was filtered through a 50 μm pore nylon mesh and resuspended in 5 ml of incubation medium.

Exclusion of the dye trypan blue (0.1% (w/v) in 0.9% (w/v) NaCl) by the isolated cells was assessed by light microscopy (Freshney, 1983).

2.8.4 Estimation of cell yield

The cell yield was estimated by transferring 100 μl of the final cell suspension into an elongated microtube which was then centrifuged (11,000 g_{av} , 30 s). The volume of the cell pellet was determined from the pellet's length, with the aid of a standard curve obtained with a dye-containing solution and cell number then estimated using a conversion factor of 6.08×10^6 cells/ml for each μl of pellet volume. This

value had been calculated over a number of experiments from values obtained from the DNA content of the cell suspension.

2.8.5 Incubation of cells

Isolated cells were resuspended in incubation medium at 0.67×10^6 cells/ml and 3 ml of suspension was dispensed into 6-well plastic tissue-culture plates (Costar, Cambridge, Mass., USA). The cells were incubated in a tissue-culture incubator (Flow Laboratories, Irvine, UK) at 37°C under 5% CO₂ (v/v) in a saturated water atmosphere. Following a 1 hour equilibration period, the cells were harvested by centrifugation (80 g_{av} , 4 min), resuspended in fresh incubation medium and incubated for a further period of time.

2.8.6 Determination of glucose utilisation by cells

The utilisation of glucose by the isolated cells was determined by measuring changes in D-glucose concentration in the incubation medium. Cells were harvested by gentle centrifugation (80 g_{av} , 4 min) and the medium stored under liquid nitrogen. The concentration of D-glucose was assessed using a diagnostic kit (Sigma Chemical Co., Poole, UK) which utilized glucose oxidation by glucose oxidase. The resultant colour-complex was assessed at 450 nm using a "Titertek multiskan" plate reader (Labsystems Ltd., Basingstoke, Hants, UK).

2.8.7 Determination of protein synthesis and secretion

Synthesis and secretion of protein was determined in isolated cells radiolabelled in triplicate 1 ml culture wells with 15 μ Ci/ml L-[4,5-³H]-leucine (specific activity > 40 Ci/mmol) for up to 3 hours in incubation medium containing no supplementary amino acids. Cells were harvested (80 g_{av} , 4 min) at the end of the incubation period, washed twice by centrifugation (6500 g_{av} , 1 min) with 1 ml of Hanks Buffer (pH 7.4) containing 5 mM- L-leucine and stored at -20°C. Non-pelleted cells were cleared from the incubation medium by centrifugation (6500 g_{av} , 1 min).

Incorporation of L-[4,5-³H]-leucine into protein was assessed by trichloroacetic acid precipitation. All procedures were performed on ice or at 4°C. Cell pellets were sonicated for 30 seconds at level 30 on a Kontes micro-ultrasonic cell disruptor (Buckard Scientific, Middlesex, UK). Bovine serum albumin (100 μ l of 0.1% w/v)

was added to 100 μl of each cell sample, with water and 400 μl of ice-cold trichloroacetic acid (20% w/v) containing 5 mM- L-leucine being added to both cell and medium samples (100 μl) to a total volume of 800 μl .

After 20 minutes, the precipitates were harvested by centrifugation (11,000 g_{av} , 5 mins), redissolved in 200 μl of 1 M-Tris (pH 8) with 200 μl of water and reprecipitated with trichloroacetic acid (20% w/v) as before. The precipitates were rinsed twice with 800 μl of trichloroacetic acid (10% w/v) containing 5 mM- L-leucine and collected by centrifugation. The final precipitates were redissolved in 200 μl of 1 M-Tris (pH 8), transferred to scintillation vials with an additional 200 μl of 1 M-Tris (pH 8) and counted for ^3H activity.

2.8.8 SDS-polyacrylamide gel electrophoresis and fluorography

The nature of proteins synthesised and secreted by the isolated cells was determined in cells labelled for 2 hours as described above, but with 100 $\mu\text{Ci/ml}$ L- $[^{35}\text{S}]$ -methionine (specific activity ≥ 1000 Ci/mmol) replacing $[^3\text{H}]$ -leucine. At the end of the incubation period, cells were harvested (6500 g_{av} , 1 min, 4°C), washed twice with Hanks buffer (pH 7.4) containing 5 mM- L-methionine and frozen in liquid nitrogen for storage at -20°C. Cell pellets were sonicated in 1 ml of water using a Kontes micro-ultrasonic cell disruptor (level 30, 15 seconds; Buckard Scientific, Middlesex, UK), dialysed (Microdialyser System 100; Pierce, Illinois, USA) and lyophilised. Medium samples were dialysed separately against water for 24 hours and then lyophilised.

The cell and medium samples were dissolved in 0.625 M-Tris (pH 7.4) containing 2% SDS, 10% glycerol, 5% mercaptoethanol and bromophenol blue to colour. The sample proteins were separated by electrophoresis in a 11% polyacrylamide gel (Laemmli, 1970) and stained with comassie brilliant blue. For fluorography, the gels were dehydrated in dimethylsulphoxide, impregnated in a saturated solution of 2,5- diphenyloxazole for 2 hours and dried onto filter paper on a slab gel dryer (Hoeffer Scientific Instruments, San Francisco, USA). X-omat RP film (Eastman Kodak, Rochester, New York, USA) was exposed to the dried gels at -70°C for 4 days before developing.

2.8.9 Determination of lactose secretion

The secretion of lactose was assessed in isolated cells labelled with 2 $\mu\text{Ci/ml}$ D-[U- ^{14}C]-glucose (specific activity ≥ 286 mCi/mmol). At the end of the incubation period, the cells were harvested by centrifugation (80 g, 4 minutes) and the incubation medium stored at -20°C .

Incorporation of D-[U- ^{14}C]-glucose into lactose was assessed by repeated precipitation in the presence of carrier lactose (Kuhn & White, 1975). To 1.2 ml of each sample was added an equal volume of ice-cold trichloroacetic acid (10% w/v) and contaminating protein removed by centrifugation (800 g_{av} , 10 min, 4°C). Supernatant (1 ml) was transferred to a stoppered glass-tube containing 100.0 ± 0.5 mg of lactose and heated, in a water bath at 70°C , until the lactose had dissolved. After cooling, the lactose was precipitated by the addition of 8 ml of ethanol-diethyl ether (3:1 v/v) with vigorous mixing, both by spatula and vortex-mixing. After 30 minutes at room temperature, the sediment was collected by centrifugation and the supernatant discarded. Three further precipitations followed, 0.5 ml of water being used in the first precipitation to dissolve the lactose, with 4 ml of ethanol-ether to precipitate it. Subsequent precipitations utilised 0.4 ml of water followed by 3 ml of ethanol-ether. The final precipitate was dissolved in 1 ml of water and 1 ml of this was counted for ^{14}C activity. The remainder of the final extract was then assessed for lactose content.

Following lactose precipitation, lactose recovery was assessed by means of σ -toluidine reduction. Samples and lactose standards, in the range of 0 to 2.88 mg/ml, were made up to 1 ml with water and placed with an equal volume of σ -toluidine, in a boiling water bath for 10 minutes. After cooling on ice, the formation of the colour complex was assessed at 635nm (Cecil 5000, Cecil Instruments, Cambridge, UK).

After correction for both the measured recovery of lactose and the carry-through of [^{14}C]-glucose in unincubated medium, the quantity of [^{14}C]-lactose present in the original sample was calculated from the final amount of precipitated [^{14}C]-lactose.

2.8.10 Electron microscopy of isolated cells

Cell suspensions were fixed for microscopy by addition of an equal volume of 0.1 M-phosphate buffer (pH 7.4) containing 0.002% (w/v) CaCl_2 and 2.5% (w/v)

glutaraldehyde. After 20 minutes at room temperature, 90% of this mixture was removed by centrifugation (80 g_{av} , 4 minutes) and an equal volume of the glutaraldehyde solution was added for 30 minutes. The cells were harvested again and rinsed once in 0.1 M-phosphate buffer in which glutaraldehyde was replaced by 2% (w/v) sucrose and left in this solution overnight at 4°C. The addition of 0.1 M-phosphate buffer containing 1% (w/v) agarose to the cells and rinsing with the sucrose-containing 0.1 M-phosphate buffer was followed by post-fixing in 1% (w/v) OsO_4 for 30 minutes. The cells were block stained in the dark with 0.5% (w/v) uranyl acetate (Agar Scientific, Stansted, UK), dehydrated by processing through increasing alcohol concentrations up to 100%, infiltrated into Araldite mix (Araldite CY212, Agar Scientific) via propylene oxide and embedded over Araldite for 48 h at 60°C.

2.9 DETERMINATION OF PROLACTIN BINDING BY ISOLATED CELLS

2.9.1 Cell harvesting

Cells were harvested from the incubation medium by gentle centrifugation and resuspended at 4×10^6 cells/ml in binding medium. If the cells had been incubated in the presence of milk fractions, these fractions were included at the same relative milk concentration in this first wash. A second wash was performed using binding medium which also contained digitonin (0.05% w/v) or saponin (0.1% w/v) if the cells were to be permeabilised. Following permeabilisation, the cells were washed once more with binding medium and resuspended at 1×10^7 cells/ml in binding medium.

2.9.2 Cell prolactin binding assay

The prolactin binding assay for isolated lactating mouse mammary cells was modified from that described by Sakai *et al* (1978).

All the reactants in the [^{125}I]-oPrl binding assay were prepared in binding medium (Table 2.1) and were added to 0.75 ml polypropylene micro-tubes in the following order:

- (1) 100 μ l of cell suspension (1×10^7 cells/ml),
- (2) 100 μ l of unlabelled oPrl (0 - 60 μ M) and
- (3) 100 μ l of [^{125}I]-oPrl (2×10^6 cpm/ml; 50 nM).

The tube-contents were vortex-mixed briefly and incubated in a water bath for 45 minutes at 37°C.

At the end of the binding reaction, the cells were sedimented by centrifugation (45 s, Eppendorf, Netheler + Hinz, GmbH) and the supernatant removed using a fine-tipped pasteur pipette. The cell pellet was rinsed once with 400 μ l of binding medium and the tube wall wiped with a cotton-tipped bud. 125 I activity of the pellet was determined and specific prolactin binding calculated by subtracting non-specific binding of [125 I]-oPrl (binding determined in the presence of excess unlabelled oPrl) from total binding (in the absence of unlabelled oPrl).

The DNA content of the cell suspension was determined in samples taken from the final cell suspension. The cell pellets were recovered by centrifugation (11,000 g_{av} , 30 s) and stored in liquid nitrogen for later analysis. Cell number was calculated using a value of 11.5 pg DNA/ cell (calculated from Lewin (1957) and Alberts *et al.* (1983)).

2.10 STATISTICS

Statistical calculations were routinely performed using MinitabTM (Release 7.2, Minitab Inc. Pennsylvania, USA). Analysis of variance was performed using Genstat 5TM (Release 2.2, Lawes Agricultural Trust, Rothamsted Experimental Station, Herts., UK).

CHAPTER THREE

EFFECTS OF MILK ACCUMULATION ON PROLACTIN BINDING IN THE LACTATING RABBIT MAMMARY GLAND

3.1 INTRODUCTION

A lactating rabbit suckles its young once a day (Zarrow *et al.*, 1965). The rate of milk accumulation in the mammary gland is constant during the 24 hour interval between sucklings, but is markedly reduced beyond this period (Calvert *et al.*, 1987). This decrease in the rate of milk secretion is the result of milk accumulation, being independent of the systemic effects arising from suckling (Calvert *et al.*, 1987) and is probably due to a combination of both physical distension and local inhibitory chemicals (Peaker, 1980; Wilde *et al.*, 1987a).

In studies in which the mammary glands of lactating rats were unilaterally sealed by teat ligation, milk stasis was accompanied by a reduction in the number of unoccupied prolactin receptors present in the glands (Hayden & Smith, 1981). It has been suggested that such changes in receptor number will affect the responsiveness of the mammary gland to circulating hormones, with resultant effects on milk secretion and mammary differentiation (Wilde *et al.*, 1990). However, little is known about the mechanism by which the changes in prolactin receptor number are induced by milk accumulation, or about the relationship of these changes to the rate of milk secretion by the gland.

The lactating rabbit was used for investigating changes in mammary gland prolactin receptor number which occur as a response to milk accumulation. The steady rate of milk secretion (Calvert *et al.*, 1987) together with a prolactin-dependent lactation (Taylor & Peaker, 1975) made this species an ideal model for such a study.

3.2 EXPERIMENTAL DESIGN AND METHODS

3.2.1 Effect of milk accumulation in the rabbit mammary gland

Six lactating Dutch-breed rabbits suckling litters of 3 to 9 young and weighing 2.0 - 2.6 kg were used during their first lactation. Starting from day 3 to 5 of lactation, the mothers were accustomed to a regime whereby they suckled their litters for 1 hour each day, followed by 23 hours of separation. Experiments were performed between days 12 and 18 of lactation (established to peak lactation in the terminology of Peaker & Taylor, 1975).

On two consecutive days immediately prior to suckling, one gland on each side of the body was sealed using tissue adhesive ("Vet-Seal", B.Braun Melsungen AG, Melsungen, Germany) following cleaning of the teat with ethanol and diethyl-ether. The animal was sacrificed by cervical dislocation immediately following a final suckling period, with the individual mammary glands being removed and placed in liquid nitrogen for storage at -20°C. Both 4.5 M-MgCl₂ stripped and non-stripped microsomal membranes were prepared from the glands and specific binding of [¹²⁵I]-oPrI was assessed by radio-receptor assay (as described in Chapter 2).

Only the three posterior pairs of glands were used in this study, as the anterior pair of glands were not always fully developed. The experimental procedure resulted in 0, 24 and 48 hours of milk accumulation, with each time point present in two separate mammary glands. The experiments were designed so that each period of milk accumulation was present on each body-half. The order in which glands were sealed varied from rabbit to rabbit, with the body halves being treated separately in terms of sample preparation, assessment of hormone binding and statistical analysis. Not all measurements were performed on all samples from every animal.

3.2.2 Microsomal membrane preparation and [¹²⁵I]-oPrI radio-receptor assay

Microsomal membranes were prepared from lactating rabbit mammary glands by differential centrifugation (Breir *et al.*, 1988), followed by two low-speed centrifugations of the CaCl₂-precipitated membranes (Shiu *et al.*, 1973), as described in Chapter 2. Endogenously bound prolactin was removed from its receptor by treatment of the isolated microsomal membranes with 4.5 M-MgCl₂ (Kelly *et al.*, 1979; Van der Gugten *et al.*, 1980) and total mammary prolactin receptor number then assessed by radio-receptor assay (Chapter 2). Binding of prolactin to free (ie. unoccupied) prolactin receptors was assessed by radio-receptor assay of the non- 4.5 M-MgCl₂ stripped membranes. Results were expressed as [¹²⁵I]-oPrI specifically bound (cpm) per mg protein and analysed using the Student's paired *t*-test.

The affinity of the prolactin receptor after each period of milk accumulation was measured in 4.5 M-MgCl₂ stripped membranes pooled from similarly treated glands. Prolactin binding was measured by radio-receptor assay in the presence of increasing concentrations (0-1.45 μM) of unlabelled oPrI and Scatchard (1949) analysis was performed on the binding data. Least-squares analysis of the initial linear

portion of the Scatchard plot was used to calculate the association constant (K_d) of the prolactin receptor.

The integrity of [125 I]-oPrl in the mammary membrane radio-receptor assay was assessed by subsequent incubation of unbound ligand in a liver membrane radio-receptor assay. After the initial mammary membrane assay was terminated by centrifugation to collect the membrane pellet, 100 μ l of supernatant was incubated for 8 hours with 500 μ g of mid-pregnant (day 17) rabbit liver microsomal membrane as described for the radio-receptor assay in Chapter 2. In addition, [125 I]-oPrl was stored, in hormone diluent buffer in the absence of membranes, alongside the initial radio-receptor assay and used as a non-treated control.

The plasma membrane content of membrane samples was assessed by measurement of 5'-nucleotidase activity (Djiane *et al.*, 1981a). This assay was performed by Ms S. Wastie, as described by Arch & Newsholme (1978) and modified by Vernon *et al.* (1983), using duplicate 10.8 μ g samples of membrane protein. The protein composition of the isolated membranes was analysed by SDS-polyacrylamide gel electrophoresis (Chapter 2) and their migration compared with molecular weight standards. Gel electrophoresis was performed by Professor W.L. Hurley.

3.3 RESULTS

Although suckling of the pups was not closely observed for fear of disturbing the mother, suckling appeared to be complete within minutes of the pups being placed with the mother.

3.3.1 Development of the membrane preparation procedure

Initially, membranes were harvested by low-speed centrifugation (2,200 g, 30 minutes, 4°C) following treatment with 4.5 M-MgCl₂. This harvesting procedure resulted in excessive protein loss ($91.5 \pm 1.4\%$ loss, mean \pm SEM, n=3) and high-speed centrifugation (30,000 g, 40 minutes, 4°C) was subsequently used to improve the yield ($77.4 \pm 0.9\%$ loss, n=6).

In the Bradford (1976) protein assay, the estimate of protein content for increasing levels of microsomal membrane was parallel to that obtained using the bovine serum albumin standard (results not shown). The apparent protein content of the membrane samples was markedly reduced following freezing (results not shown) and for this reason the protein content of all membrane samples was assessed prior

and for this reason the protein content of all membrane samples was assessed prior to freezing.

The 5'-nucleotidase activity of both 4.5 M-MgCl₂ stripped and non-stripped membranes from suckled glands was similar to that of membranes prepared from glands in which milk had accumulated (Figure 3.1). Although 4.5 M-MgCl₂ stripped membranes tended to have a lower 5'-nucleotidase activity than non-stripped membranes (0.81 ± 0.47 vs. 1.20 ± 0.23 nmol/min/mg protein, stripped vs. non-stripped, mean \pm SEM, n=3), this difference was not statistically significant ($P > 0.05$).

When resolved by SDS-polyacrylamide gel electrophoresis, the apparent density of the protein bands corresponding to milk caseins was similar in microsomal membranes prepared from suckled and milk-accumulated glands. Treatment of microsomal membranes with 4.5 M-MgCl₂ did not affect the apparent density of the casein bands in the membranes, although band densities differed in membranes isolated in separate preparations (Plate 3.1).

3.3.2 Characterisation of the radio-receptor assay

Specific binding of ovine prolactin to non- 4.5 M-MgCl₂ stripped mammary microsomal membranes was linear in the range of 0 - 200 μ g membrane protein (Figure 3.2), with specific binding to 4.5 M-MgCl₂ stripped membranes being linear up to 100 μ g of membrane protein (Figure 3.2). A quantity of 90 μ g of membrane per assay tube was adopted as the optimal amount of protein for use in this radio-receptor assay, utilising a modest amount of membrane protein whilst achieving high levels of specific [¹²⁵I]-oPrl binding.

In suckled mammary glands, non-specific binding of [¹²⁵I]-oPrl was $52.4 \pm 4.4\%$ (mean \pm SEM, n=8) of total radioactivity bound for 4.5 M-MgCl₂ stripped membranes and $57.9 \pm 4.5\%$ (n=7) for non-stripped membranes, with $7.1 \pm 1.5\%$ and $14.1 \pm 2.9\%$ of the total radioactivity being specifically bound respectively. In 200 μ g of mid-pregnant (day 17) rabbit liver membranes, which were used as a positive control in the radio-receptor assay, $59.3 \pm 1.3\%$ (n=6) of the total bound radioactivity was non-specific, with specific binding being $11.0 \pm 1.2\%$ of total radioactivity added. The [¹²⁵I]-oPrl specifically bound to the mammary membranes was displaced by increasing concentrations of unlabelled ovine prolactin, with there being no displacement by an excess (4.35 μ M) of unlabelled porcine insulin

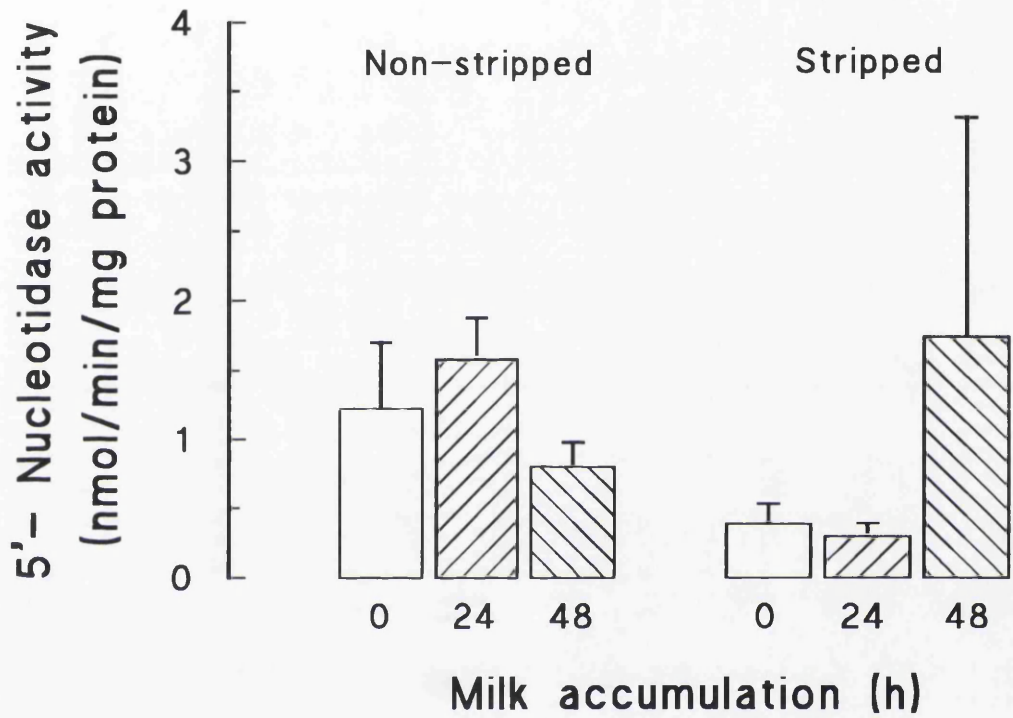
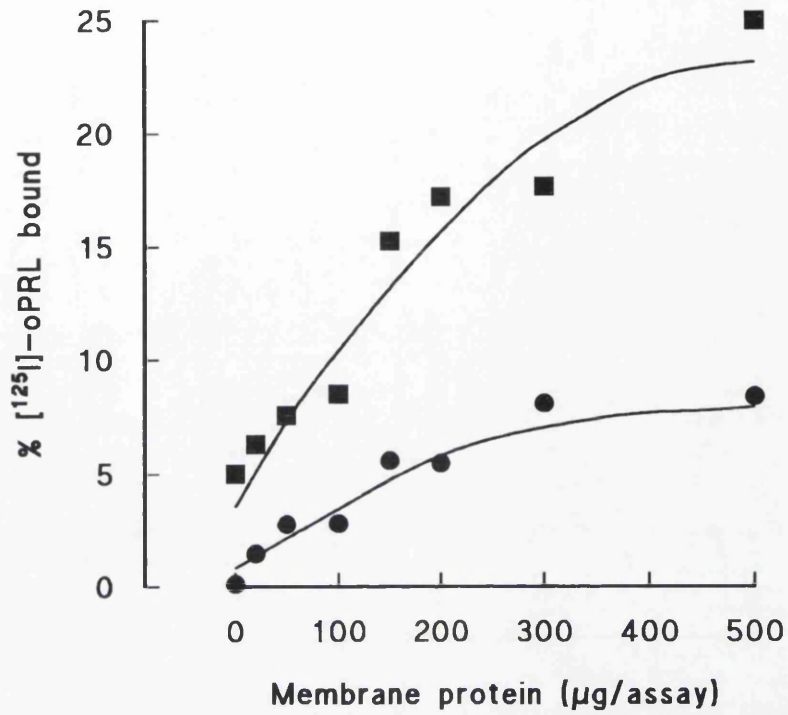


Figure 3.1. 5'-Nucleotidase enzyme activities of 4.5 M-MgCl₂ stripped and non-stripped microsomal membranes prepared from lactating rabbit mammary glands subject to 0, 24 and 48 hours of milk accumulation. Values are means + half the range for duplicate measurements.

a)



b)

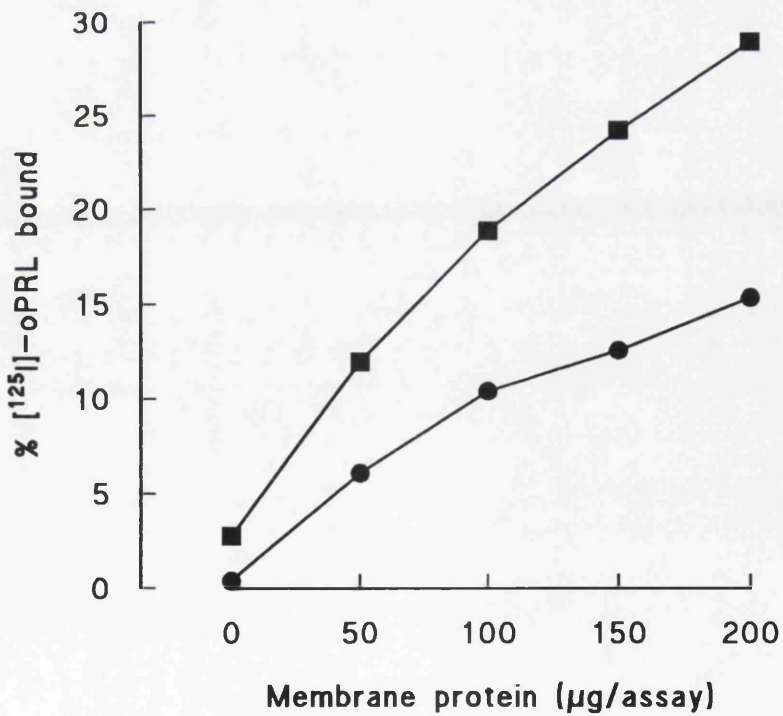


Figure 3.2. Effect of increasing levels of membrane protein in the radio-receptor assay on total (■) and specific (●) binding of $[^{125}\text{I}]\text{-oPrI}$, expressed as a percentage of total radioactivity, to a) non- 4.5 M-MgCl₂ stripped and b) 4.5 M-MgCl₂ stripped microsomal membranes.

Plate 3.1 SDS-polyacrylamide gel electrophoresis of 4.5 M-MgCl₂ stripped and non-stripped microsomal membranes prepared from lactating rabbit mammary glands subject to 48 h and 0 h of milk accumulation. Bands corresponding with molecular weight markers (mwm) are also shown. WAP - whey acidic protein.

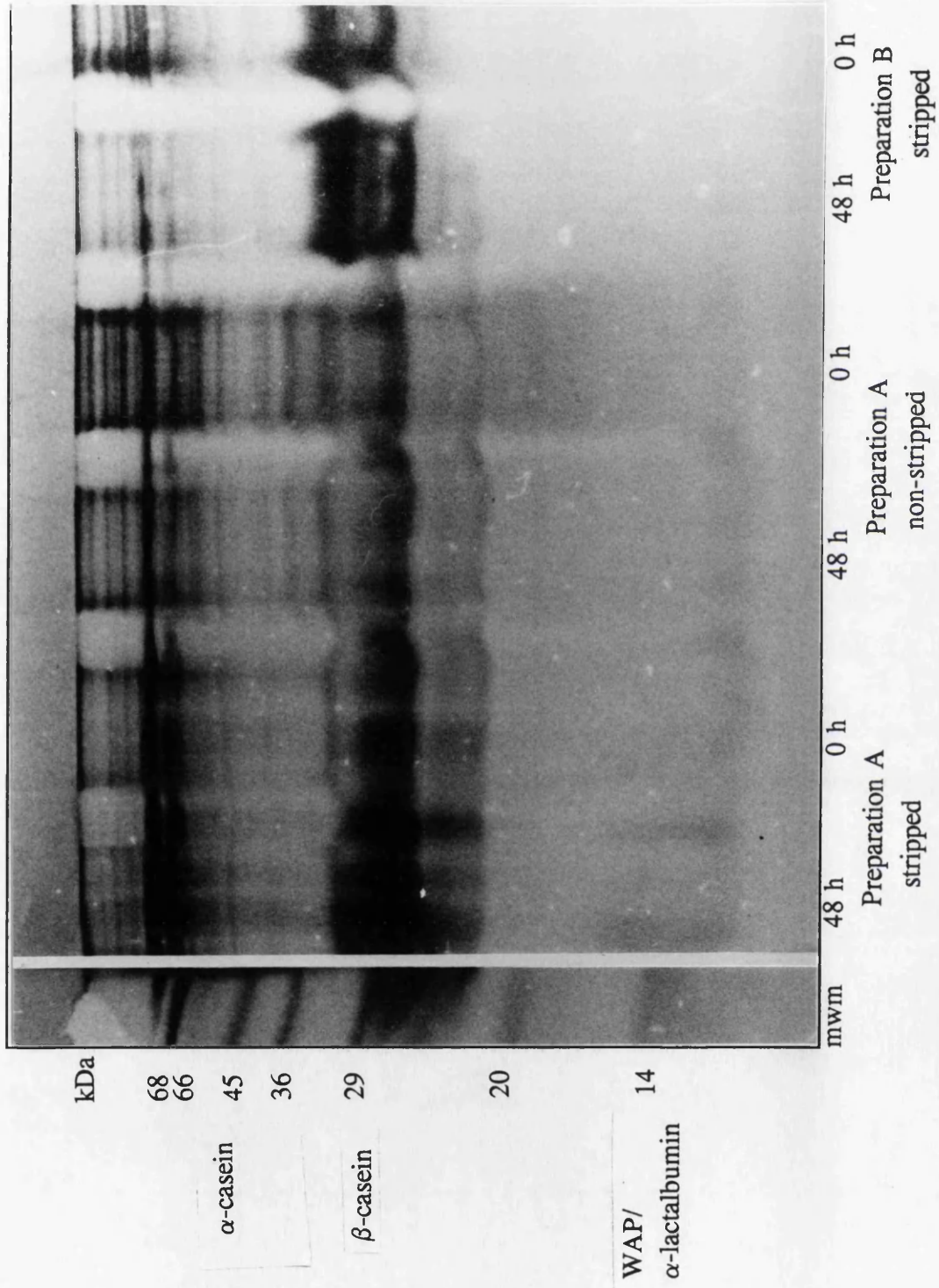


Plate 3.1

(Figure 3.3). The association constant (K_d) of the prolactin receptor for [125 I]-oPrl, as determined by Scatchard analysis using 4.5 M-MgCl₂ treated membranes prepared from suckled and non-suckled glands was $1.89 \pm 0.38 \times 10^9 \text{ M}^{-1}$ ($n=11$, Figure 3.4).

The incubation of [125 I]-oPrl with membranes prepared from glands subject to differing periods of milk accumulation did not influence specific binding of the unbound ligand in a subsequent liver radio-receptor assay ($n=3$) (Figure 3.5).

3.3.3 The effects of milk accumulation on prolactin receptor number

The effect of milk accumulation on [125 I]-oPrl binding to 4.5 M-MgCl₂ stripped membranes was assessed using 8 sets of glands from 6 animals. There was a $19.5 \pm 3.7\%$ reduction ($P=0.01$) in specific [125 I]-oPrl binding after 24 hours of milk accumulation ($86,508 \pm 18,722$ vs. $58,329 \pm 11,819$ cpm/mg protein, 0 h vs. 24 h) (Figure 3.6). After 48 hours of milk accumulation, there was no further change in specific prolactin binding ($59,825 \pm 12,912$ cpm/mg protein), although the level of binding at this time was still significantly reduced in comparison to that seen in the suckled (0 h) glands (by $30.2 \pm 2.3\%$, $P<0.01$).

The effect of milk accumulation on the binding of [125 I]-oPrl to non- 4.5 M-MgCl₂ stripped membranes was assessed using 7 sets of glands from 5 animals (Figure 3.6), with binding representing $48 \pm 7\%$ of that to the stripped membranes in the suckled glands. There was no significant change in specific prolactin binding to the non-stripped membranes following 24 hours of milk accumulation ($36,422 \pm 6273$ vs. $41,270 \pm 5134$ cpm/mg protein, 0 h vs. 24 h). However, there was a slight but non-significant reduction in prolactin binding after 48 hours of milk accumulation ($27,535 \pm 4602$ cpm/mg protein, $P=0.071$), when compared to the suckled (0 h) glands.

Despite considerable variation between individual experiments, there was no significant effect of milk accumulation on the affinity (K_d) of the prolactin receptor when assessed by Scatchard analysis. In membranes prepared from suckled glands, an estimate of 258 ± 124 fmol oPrl binding sites per mg protein was obtained ($n=4$). However in the analysis shown in Figure 3.4, it was not possible to quantify changes in receptor site number with milk accumulation, since membranes were pooled from glands of different animals.

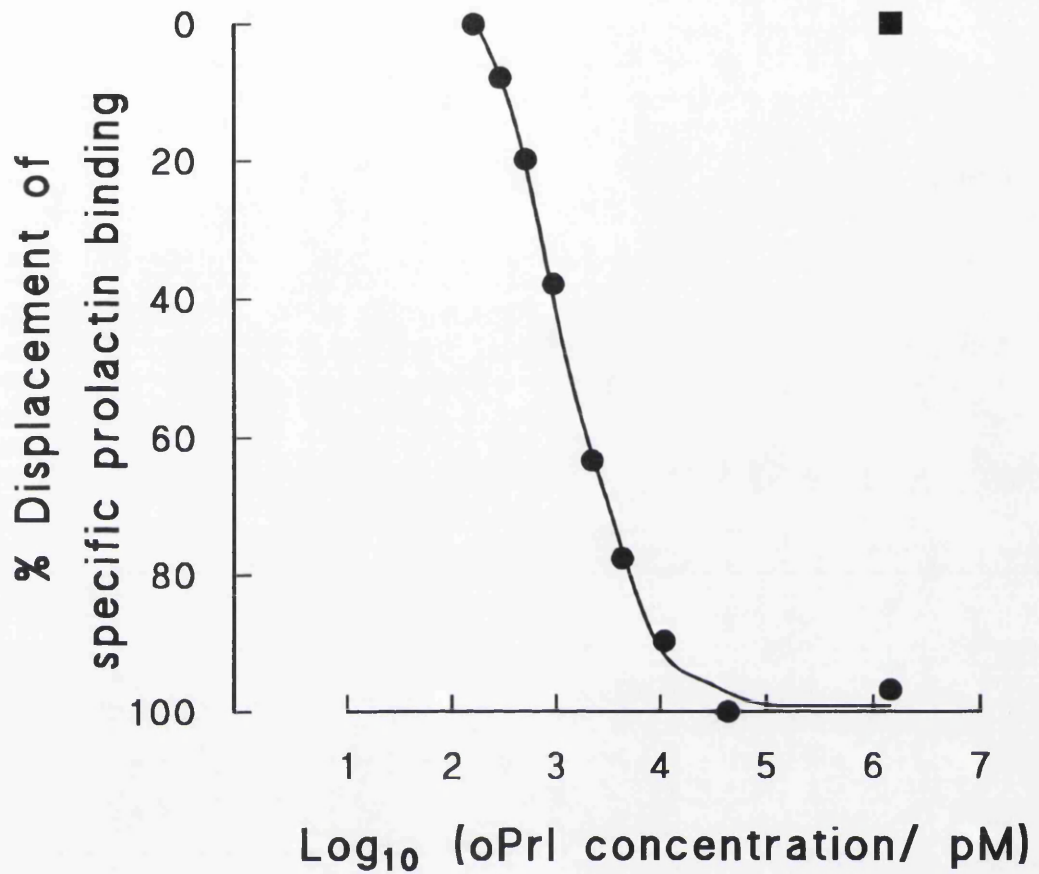


Figure 3.3. Effect of increasing levels of unlabelled oPrI (●) in the radio-receptor assay on the specific binding of [¹²⁵I]-oPrI to 4.5 M-MgCl₂ stripped microsomal membranes prepared from suckled lactating rabbit mammary glands. Specific [¹²⁵I]-oPrI binding in the presence of an excess (1.45 μM) of unlabelled porcine insulin is also shown (■).

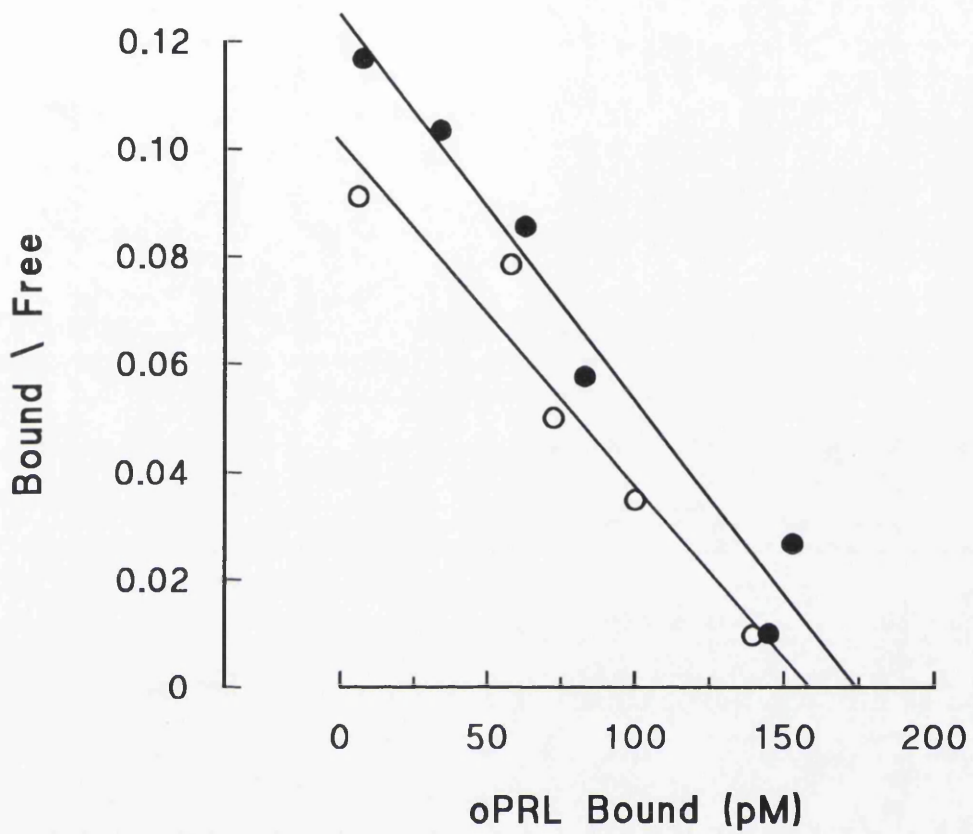


Figure 3.4. Scatchard (1949) analysis of [¹²⁵I]-oPrl binding to 4.5 M-MgCl₂ stripped membranes prepared from lactating rabbit mammary glands subject to 0 (○) and 24 (●) hours of milk accumulation. The association constant (K_d) of the prolactin receptor was calculated to be $1.89 \pm 0.38 \times 10^9 \text{ M}^{-1}$.

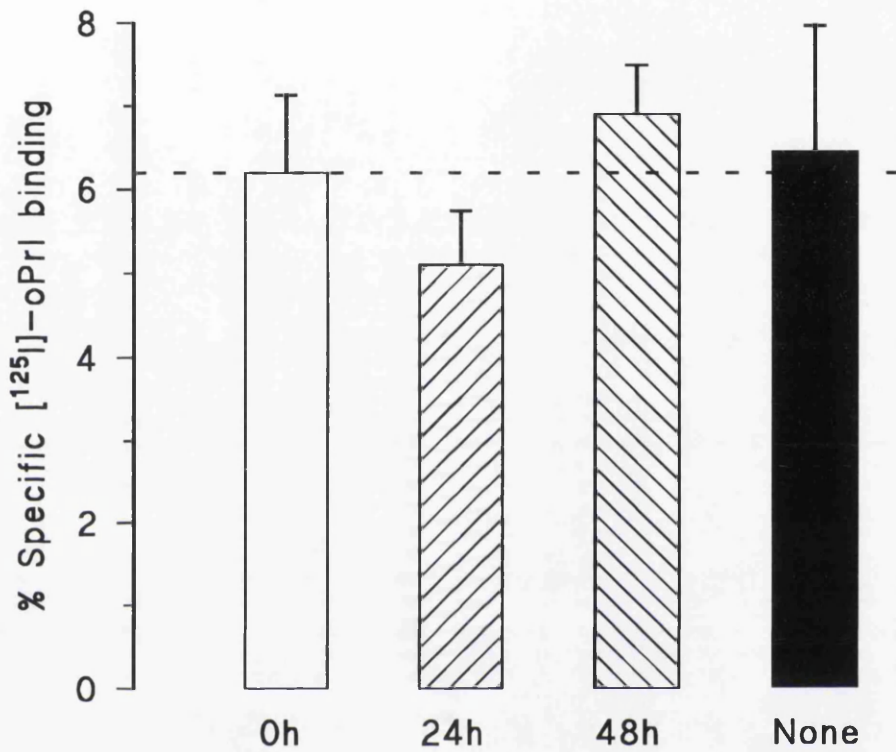


Figure 3.5. Specific binding of [125 I]-oPrI in a liver membrane radio-receptor assay following a previous incubation in a radio-receptor assay in either the presence (open or hashed bars) or absence (solid bar) of 4.5 M-MgCl₂ stripped mammary membranes. Membranes were prepared from lactating rabbit mammary glands subject to 0, 24 and 48 hours of milk accumulation. Values are means + SEM.

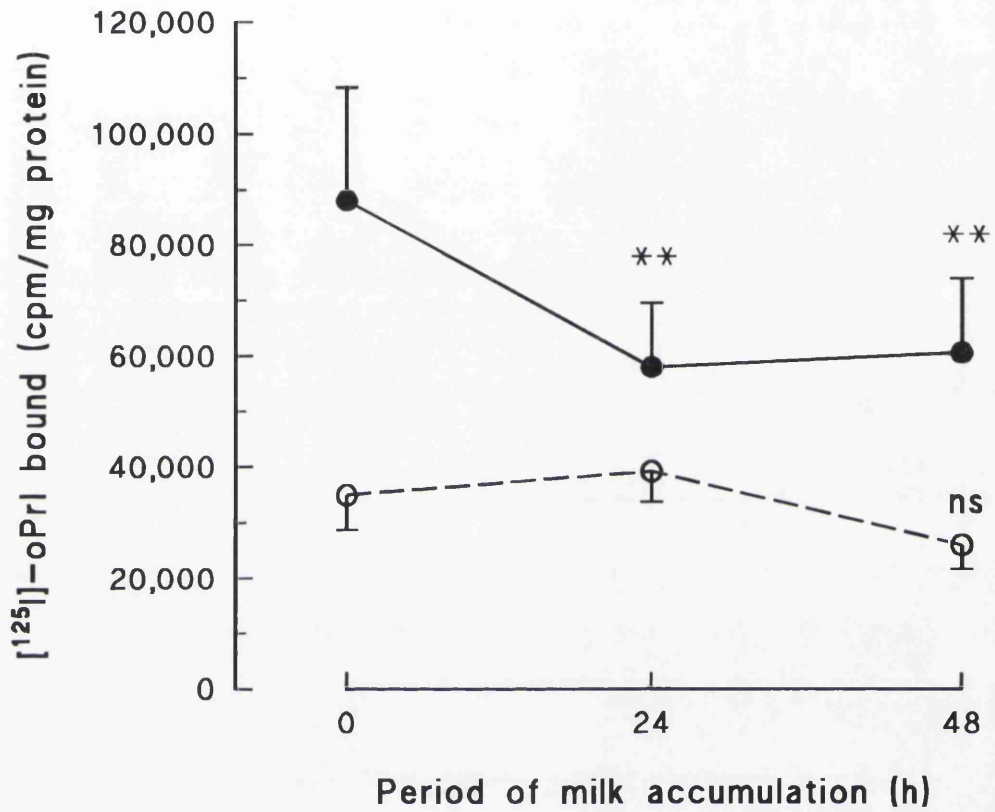


Figure 3.6. Specific [^{125}I]-oPrl binding to 4.5 M-MgCl₂ stripped (●) and non-stripped membranes (○) prepared from lactating rabbit mammary glands subject to 0, 24 and 48 hours of milk accumulation. Values are means \pm SEM. (** - $P \leq 0.01$; ns - not significant, when compared to 0 h values).

3.4 DISCUSSION

The effects of milk accumulation on prolactin binding by the mammary gland were investigated using the lactating rabbit as a model. Sealing individual mammary glands of a suckled animal allowed effects arising from engorgement of the gland with milk to be separated from those caused by withdrawal of the suckling stimulus. The once daily suckling regime utilised here for the lactating rabbit does not affect normal development of the litter (Zarrow *et al.*, 1965).

3.4.1 Characterisation of rabbit mammary prolactin binding

Mammary prolactin binding was assessed using microsomal membranes prepared from glands subject to differing periods of milk accumulation. Treatment of microsomal membranes with 4.5 M-MgCl₂ removes endogenously bound prolactin from its receptor (Kelly *et al.*, 1979; Van der Gugten *et al.*, 1980), enabling the total number of receptors to be assessed by radio-receptor assay. Despite the use of high-speed centrifugation to harvest the membranes following this treatment, a considerable amount of protein was lost (23% recovery) when compared to the study of Kelly *et al.* (1979), which reported a 68-76% recovery of membrane. It has been suggested that the protein loss following 4.5 M-MgCl₂ treatment is not random (Hayden & Smith, 1981) and the low rate of protein recovery reported here possibly reflects the reduced contamination of the microsomal membranes by casein in this study.

It was necessary to remove excess casein from the isolated membranes, in order to prevent an over-estimate of membrane content in the protein assay and a resultant under-estimate of [¹²⁵I]-oPrl binding when expressed on a unit protein basis. Excess casein was removed by centrifugal harvesting of the CaCl₂-precipitated membranes, under conditions in which the casein was not precipitated. The enzyme 5'-nucleotidase is a marker for the plasma membrane and the activity of this enzyme can be used to quantify the plasma membrane content of isolated membranes (Djiane *et al.*, 1981a). The similar estimates of both 5'-nucleotidase activity and casein contamination in the membranes from suckled and milk-accumulated glands demonstrated that excess milk protein was removed to a similar extent by the membrane preparation procedure. Consequently, changes in mammary prolactin binding could be accurately determined on a unit protein basis.

The affinity of the prolactin receptor, as assessed by Scatchard analysis, was not affected by accumulation of milk in the mammary gland ($K_a = 1.89 \times 10^9 \text{ M}^{-1}$).

This is in agreement with previous studies in the rabbit which, with the exception of that by Perry & Jacobs (1978), have shown constancy in the affinity of prolactin for its receptor throughout lactation ($K_a = 2.5 - 3.2 \times 10^9 \text{ M}^{-1}$) (Djiane *et al.*, 1977) and following cessation of suckling ($K_a = 1.75 \times 10^9 \text{ M}^{-1}$) (Jones & Parker, 1983). Although two separate forms of prolactin receptor (Kelly *et al.*, 1992; Sakai & Ike, 1987) are differentially expressed in the mammary gland (Jahn *et al.*, 1991), their similar affinities prevent receptor type identification from Scatchard analysis of [¹²⁵I]-oPrI binding data (Lesueur *et al.*, 1991). The consistency of the receptor association constant reported here indicates that the affinity of the prolactin receptor for the circulating hormone is not affected by local factors resulting from milk accumulation in the gland. In addition, the effects of milk accumulation on prolactin binding were not a consequence of an action, proteolytic or otherwise, on the prolactin tracer in the radio-receptor assay, as the specific binding of the tracer in a subsequent radio-receptor assay was not affected.

3.4.2 Effect of milk accumulation on mammary prolactin binding

The total number of prolactin receptors present in the mammary gland was significantly reduced after 24 hours of milk accumulation. This reduction preceded the locally-induced changes in the rate of milk accumulation seen after this time (Calvert *et al.*, 1987). The reduction in receptor number is unlikely to be a consequence of a change in mammary blood flow, for there is no difference in cardiac output or mammary blood flow either shortly before or after suckling (Ota & Peaker, 1979) and so must result from other local effects arising from engorgement of the gland with milk.

Acute local control of milk secretion is exerted by a secreted milk constituent (Peaker & Wilde, 1988), which is thought to act as a feedback inhibitor of lactation (FIL) (Addey *et al.*, 1991b). It is possible that FIL, in addition to inhibiting milk synthesis and secretion, is also responsible for the milk accumulation-induced changes in hormone receptor number. The differentiated state of lactating rabbit mammary tissue, as indicated by lactogenic enzyme activities, can be rapidly reversed by the FIL-containing 10-30 kDa goat whey fraction *in vivo* (Wilde *et al.*, 1988b). The loss of differentiation is either accompanied, or mediated, by a reduction in mammary gland prolactin receptor number, as has been demonstrated for incompletely milked goats (McKinnon *et al.*, 1988) and unilaterally-occluded, suckled rats (Hayden &

Smith, 1981). It was not possible in the study reported here, to determine whether the milk accumulation-induced reduction in prolactin receptor number was a consequence of, or caused, the reduced rate of milk secretion seen beyond 24 hours of milk accumulation.

It is also possible that the milk accumulation-induced reduction in prolactin receptor number was a consequence of physical distension in the mammary gland. It is known that cell shape and structure is important in promoting mammary cell differentiation *in vitro* (Hauptle *et al.*, 1983) and it is possible that alterations in cellular morphology could affect receptor synthesis and degradation. However, such an effect of physical distension would appear to separate the gland's response to milk accumulation in the rabbit from that observed in the goat where, during periods of milk accumulation when rising intra-mammary pressure should not affect milk secretion (Fleet & Peaker, 1978), mammary prolactin sensitivity or receptor number is affected by the frequency of milk removal (Knight *et al.*, 1990a; McKinnon *et al.*, 1988). There are other reasons for believing that physical distension does not affect hormone receptor number and these will be discussed in Chapter 4.

In contrast to the milk accumulation-induced changes in total mammary prolactin receptor, there was no accompanying change in the number of free, or unoccupied, prolactin receptors. In lactating rabbits subjected to a complete removal of the suckling stimulus, the number of free prolactin receptors in the mammary gland does not change significantly until day 10 of involution (Jones & Parker, 1983). However, in studies of the type performed by Jones and Parker (1983), it is not possible to separate the local effects of milk accumulation from the systemic effects arising from the removal of the suckling stimulus.

The physiological significance of the unoccupied prolactin receptors is uncertain, with $48.0 \pm 7.5\%$ ($n=6$) of receptors remaining unoccupied in the suckled (0 h) glands in this study. That these receptors were not occupied following the post-suckling increase in circulating prolactin (McNeilly & Friesen, 1978), suggests that they were not available to the circulating hormone and therefore not located on the surface of the mammary epithelial cell. In bromocriptine-treated rabbits, 20% of prolactin receptors remain unoccupied following injection with a large quantity (3 mg) of exogenous prolactin (Djiane *et al.*, 1979). It is possible that these unoccupied receptors serve as an internal reservoir, which may under certain conditions replace receptors removed from the cell surface.

A reduction in the number of prolactin receptors in the mammary gland should reduce its sensitivity to the circulating hormone. Such changes would be expected to affect milk secretion and mammary differentiation, with the response to prolactin being dose-dependent *in vitro* (Djiane *et al.*, 1987) with casein secretion in rabbit mammary tissue being rapidly affected by prolactin addition *in vitro* (Seddiki & Ollivier-Bousquet, 1991).

The reduction in prolactin receptor number after 24 hours of milk accumulation implies that the receptors within the mammary gland are subject to rapid control. Prolactin receptor number was lower in the non-suckled (24 h) glands just one hour after milk-removal from the suckled (0 h) glands, with both sets of glands being exposed to the same stimuli prior to this final suckling period.

The mechanism by which rapid changes in prolactin receptor number are brought about by accumulation of milk in the gland is not clear and the explanations centre on the control of receptor synthesis and degradation. When secretory cell differentiation is less than that achieved in normal lactation, there is intracellular degradation of milk caseins (Wilde *et al.*, 1989a) and this can be induced by the 10-30 kDa goat whey fraction *in vitro* (Wilde *et al.*, 1989a). It is possible that the milk accumulation-induced reduction in prolactin receptor number was a consequence of an increased degradation of receptors which had been internalised (Djiane *et al.*, 1981a) as a consequence of the suckling induced surge in circulating prolactin (McNeilly & Friesen, 1978).

It is also possible that milk removal could induce receptor synthesis within the mammary gland, with receptor number then declining as milk accumulates. However, the response interval is too acute for control to be exerted by gene transcription (Vonderhaar & Ziska, 1989) and other mechanisms, such as that exerted by FIL on protein synthesis *in vitro* (Rennison *et al.*, 1993), are likely to be operating. The control of prolactin receptor synthesis is explored further in Chapter 6.

3.5 CONCLUSION

In lactating rabbit mammary tissue, there is a significant reduction in prolactin receptor number as milk accumulates within the gland. This reduction precedes the change in milk secretion observed after 24 hours of milk accumulation. The reduction in hormone receptor number could influence the sensitivity of the mammary gland to circulating hormones and affect mammary cell differentiation and milk yield.

CHAPTER FOUR

EFFECT OF GOAT MILK FRACTIONS ON HORMONE BINDING IN THE LACTATING RABBIT MAMMARY GLAND

4.1 INTRODUCTION

In the rabbit, the rate of milk accumulation is constant during the 24 hour period between sucklings (Calvert *et al.*, 1987). It was shown in the previous chapter, that there is a marked reduction in the total number of prolactin receptors in the mammary gland at the end of this suckling interval. This reduction precedes the locally-induced decrease in the rate of milk accumulation (Calvert *et al.*, 1987), which is caused by a combination of both physical distension and local chemical inhibitory-factors (Peaker, 1980; Wilde *et al.*, 1987a). Milk accumulation and lactogenic enzyme activities are inhibited in the lactating rabbit mammary gland by a 10-30 kDa goat whey protein fraction, both *in vivo* and *in vitro* (Wilde *et al.*, 1987a; Wilde *et al.*, 1988b). The feedback inhibitor of lactation (FIL) is the active constituent of this whey fraction (Addey *et al.*, 1991b) and is thought to mediate the local control of milk secretion.

This study investigated whether FIL, in addition to regulating milk secretion (Rennison *et al.*, 1993), is also responsible for the local reduction in mammary gland prolactin receptor number which results from milk accumulation.

4.2 EXPERIMENTAL DESIGN AND METHODS

4.2.1 Intraductal injection of milk fractions and the determination of mammary hormone binding

The study investigated changes in prolactin binding, following intraductal injections of goat whey fractions into the lactating rabbit mammary gland.

First-lactation Dutch-breed rabbits, suckling litters of 1 - 6 pups and weighing 1.8 - 2.6 kg were used at peak lactation, after being accustomed to a once-daily suckling regime (Chapter 3). A rabbit suckling one pup was included in an experimental group in which the effects of the 10-30 kDa goat whey fraction were investigated over 7 hours, because it was apparent from the observation of suckling and from the mammary tissue that a consistent suckling stimulus had been applied to all glands.

The 10-30 kDa goat whey fraction and FIL were prepared from goat's milk collected at mid-lactation (week 13 to week 30) as described in Chapter 2, reconstituted at 20x relative to their milk concentration in carrier solution (10 mM-Hepes at pH 6.7 containing 0.3 M-sucrose) and sterilised through a 0.45 μ m pore filter (Gelman Sciences, Southampton, UK). The presence of FIL in the 10-30 kDa goat whey fraction was verified by anion exchange chromatography, as described for the isolation of caprine milk fractions (Chapter 2). Resolution of the third eluted protein peak, as shown in Figure 4.1, was taken to indicate the presence of FIL (Addey *et al.*, 1991b).

Immediately following the end of a suckling period, the animals were anaesthetised using "Hypnorm" (0.5 ml/kg, fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml, Janssen Pharmaceutical Ltd., Oxford, UK) administered intramuscularly. The test solution (10-30 kDa goat whey fraction or FIL) was introduced by intraductal injection into the three posterior mammary glands on one side of the body, with each gland receiving 1.0 - 1.2 ml (0.2 - 0.25 ml/duct, 6 ducts/gland) through blunt-ended 26-gauge needles. The contralateral glands received carrier solution alone. The animal was returned to its cage in order to recover and was then sacrificed by cervical dislocation, either 7 or 12 hours after treatment with the 10-30 kDa goat whey fraction or 12 hours after FIL-treatment.

The individual mammary glands were dissected out, placed in liquid nitrogen for storage at -20°C and 4.5 M-MgCl₂ stripped and non-stripped microsomal membranes prepared as described in Chapter 2. Specific binding of [¹²⁵I]-oPrI and [¹²⁵I]-IGF-I to the prepared membranes was assessed by radio-receptor assay (Chapter 2). The association constant (K_d) of the prolactin receptor was determined by Scatchard (1949) analysis of [¹²⁵I]-oPrI binding to 4.5 M-MgCl₂ stripped membranes pooled from the FIL-treated or carrier-treated glands of individual animals, as described in Chapter 3. Results were expressed as either [¹²⁵I]-oPrI or [¹²⁵I]-IGF-I specifically bound (cpm) per mg protein. The effects of FIL were compared by analysis of variance, whilst each 10-30 kDa goat whey fraction treated-animal was regarded as a separate experimental observation. In the latter case, data for membranes pooled from similarly-treated glands were analysed by the Student's paired *t*-test.

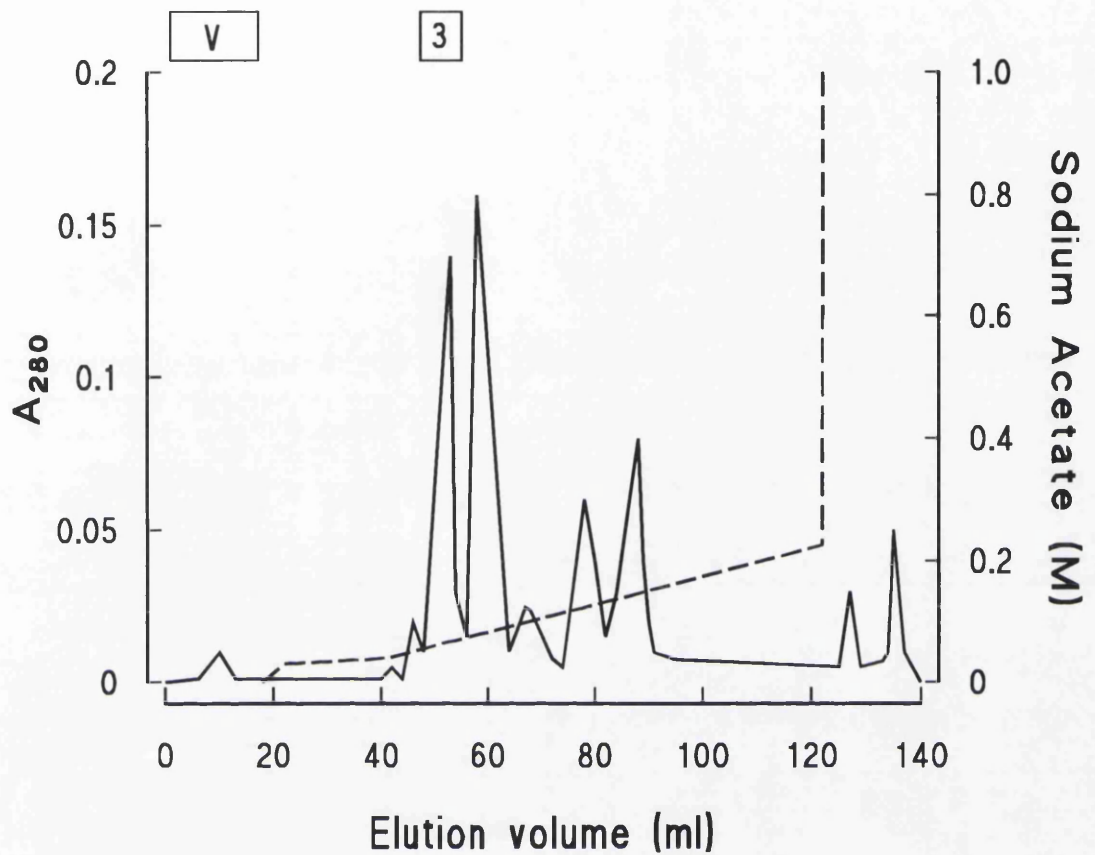


Figure 4.1. Resolution of the 10-30 kDa goat whey fraction by anion exchange chromatography, using a Mono Q HR 10/10 column (FPLC System, Pharmacia, Uppsala, Sweden), 10 mM-bistris propane pH 7.0 and a 0 - 1.0 M-sodium acetate gradient. FIL was collected as the third resolved fraction (3). V - Void volume containing material not bound by the column.

4.2.2 Direct effect of whey fractions on prolactin binding in the radio-receptor assay

Inhibition of prolactin binding to its receptor by the 10-30 kDa whey fraction, or FIL, was measured in a radio-receptor assay with 4.5 M-MgCl₂ stripped membranes prepared from suckled rabbit mammary glands (Chapter 3). This assay was performed as described in Chapter 2, with the milk fractions present in an additional 100 µl of membrane dilution buffer (pH 7.4) at a final assay concentration of 1.75x relative to that found in milk.

4.3 RESULTS

Milk fractions were introduced into the mammary glands of lactating rabbits in amounts estimated to be twice that normally present within the gland at the end of a normal (24 h) suckling interval (Calvert *et al.*, 1987). The 10-30 kDa goat whey protein fraction was present in the 20x concentrated test solution at 0.90 - 2.45 mg protein/ml, whilst FIL was present at a concentration of 8.7 - 12.0 µg protein/ml.

4.3.1 Prolactin binding

Specific prolactin binding to 4.5 M-MgCl₂ stripped mammary microsomal membranes was reduced 12 hours after the introduction of the 10-30 kDa goat whey fraction (23.9 ± 5.5% reduction, $P < 0.05$, $n = 3$ animals, Figure 4.2) into the gland. This effect was not apparent 7 hours after the whey fraction was introduced into the gland ($n = 2$ animals, Figure 4.2). The effect of the 10-30 kDa goat whey fraction on prolactin binding to non-stripped mammary membranes was not assessed.

There was a significant reduction in specific prolactin binding in both 4.5 M-MgCl₂ stripped (26.3 ± 3.4% reduction, $P < 0.001$, $n = 4$ animals) and non-stripped mammary membranes (21.0 ± 5.6% reduction, $P < 0.01$) 12 hours after FIL was introduced into the gland (Figure 4.3, Table 4.1). The proportion of the total receptors (i.e. stripped) which remained unoccupied (i.e. non-stripped) was not affected by treatment with FIL (38 ± 3% vs. 41 ± 2% unoccupied, carrier vs. FIL, $P > 0.05$). When expressed with respect to mammary gland DNA content, specific [¹²⁵I]-oPrl binding to 4.5 M-MgCl₂ stripped membranes tended to be lower in FIL-treated glands when compared to carrier-treated glands (14,309 ± 563 vs. 11,029 ± 1384 cpm/mg DNA; carrier vs. FIL), although this reduction was not statistically significant ($P = 0.086$, analysis of variance).

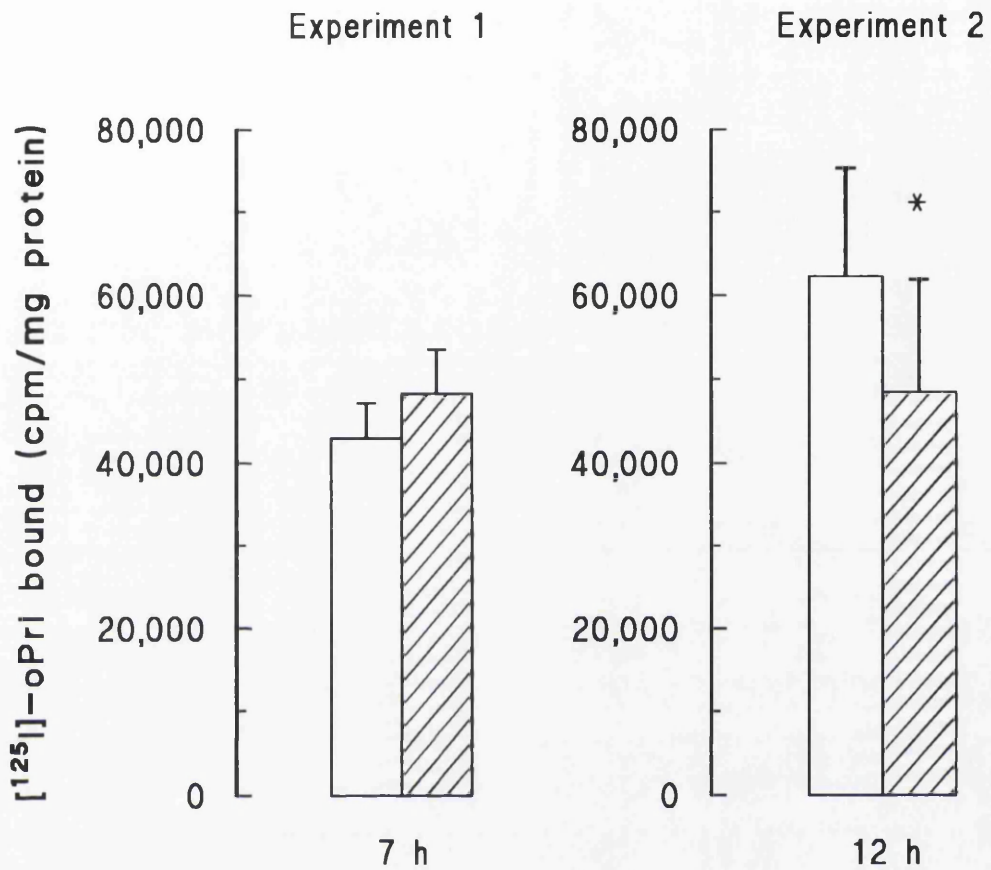


Figure 4.2. Specific [¹²⁵I]-oPrl binding to 4.5 M-MgCl₂ stripped microosomal membranes prepared from lactating rabbit mammary glands 7 (experiment 1) or 12 hours (experiment 2) after the intraductal injection of the 10-30 kDa goat whey fraction (hatched bars) or the carrier (open bars) into the gland. Values are means + half the range for experiment 1 and means + SEM for experiment 2. (* *P* < 0.05, when compared by Student's paired *t*-test with the values for the carrier-treated glands).

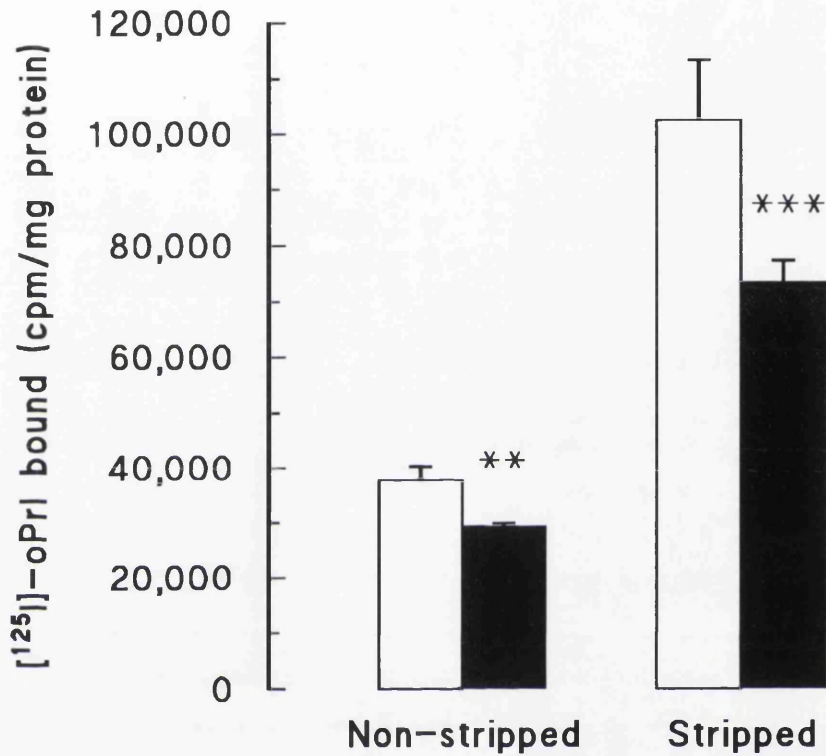


Figure 4.3. Specific binding of [¹²⁵I]-oPrl to 4.5 M-MgCl₂ stripped and non-stripped microsomal membranes prepared from lactating rabbit mammary glands 12 hours after the intraductal injection of FIL (solid bars) or carrier (open bars) into the glands. Values are means + SEM. (** *P* < 0.01; *** *P* < 0.001, when compared by analysis of variance with the values for the carrier-treated glands).

Table 4.1. Effect of a) treatment with carrier or FIL, b) body side or c) gland pair on total (stripped) and free (non-stripped) prolactin and IGF-I binding in membranes prepared from lactating rabbit mammary glands. Results were analysed by analysis of variance and values are least-square means. The three posterior pairs of glands were numbered from head to tail. sed - standard error of difference, d.f. - degrees of freedom.

a)		Specific binding (cpm/mg protein)					
Treatment	Carrier	FIL	sed	d.f.	residual	P	
Total Prl	102,139	73,539	4856	1	11	<0.001	
Free Prl	36,815	29,403	2188	1	11	<0.01	
IGF-I	85,926	76,057	4135	1	10	<0.05	
b)		Specific binding (cpm/mg protein)					
Side	Left	Right	sed	d.f.	residual	P	
Total Prl	90,266	85,412	5698	1	18	0.406	
Free Prl	36,249	29,969	2331	1	18	0.616	
IGF-I	85,042	76,940	7697	1	16	0.324	
c)		Specific binding (cpm/mg protein)					
Gland	1	2	3	d.f.	residual	P	
Total Prl	89,656	93,173	80,689	2	15	0.272	
Free Prl	34,714	33,448	31,165	2	15	0.530	
IGF-I	72,338	84,790	89,900	2	13	0.108	

Table 4.1.

Introduction of FIL into the mammary gland did not affect the affinity of [¹²⁵I]-oPrl binding to 4.5 M-MgCl₂ stripped microsomal membranes with measurements being made in 2 of the 4 FIL-treated animals (Figure 4.4). The association constant (K_a) of the receptor was calculated by Scatchard analysis to be $3.35 \pm 0.54 \times 10^9 \text{ M}^{-1}$ (n=4) in both FIL- and carrier-treated glands. Total prolactin receptor number in the carrier-treated glands was reduced from $210 \pm 1 \text{ fmol oPrl bound/mg protein}$ (mean \pm half the range) to $121 \pm 27 \text{ fmol oPrl bound/mg protein}$ in the FIL-treated glands.

Specific prolactin binding to both stripped and non-stripped mammary membranes, when assessed by analysis of variance, was not affected by either body side or gland position ($P > 0.05$, Table 4.1).

4.3.2 IGF-I binding

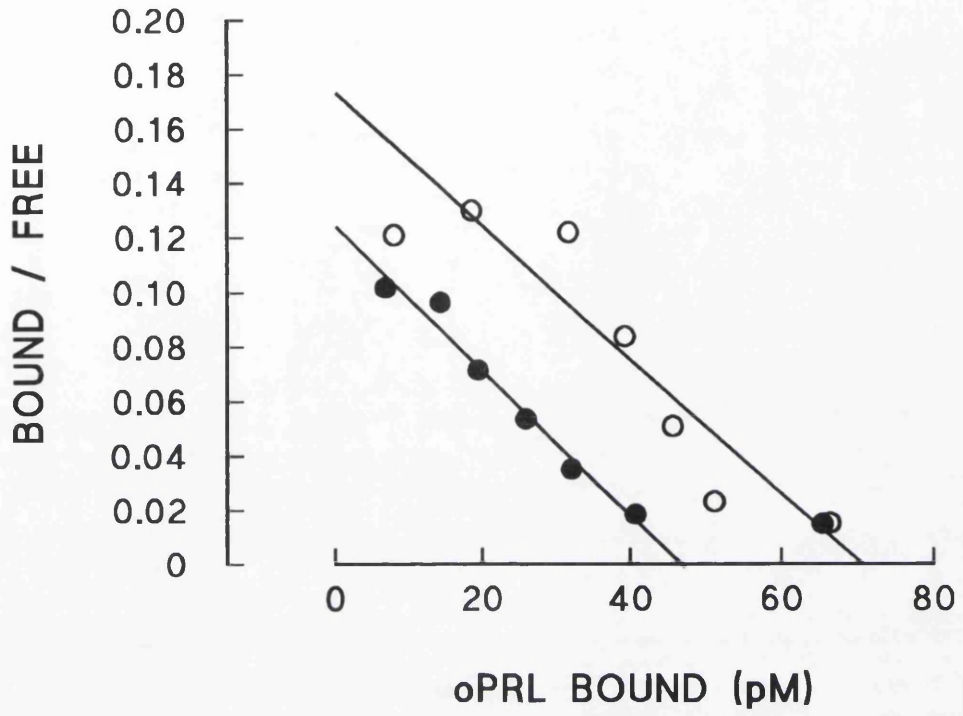
In 4.5 M-MgCl₂ stripped membranes prepared from carrier-treated lactating rabbit mammary glands, the specific binding of IGF-I was $14.9 \pm 1.5\%$ (n=11) of total radioactivity added, with non-specific binding representing $38.7 \pm 2.0\%$ of the total radioactivity bound. By comparison, non-specific binding of IGF-1 to 200 μg of mid-pregnant (day 17) rabbit liver membrane represented 57 - 60% (n=2) of the total radioactivity bound, with specific binding of 7.4 - 8.2% of the total radioactivity added.

Intraductal injection of FIL reduced specific IGF-I binding to stripped mammary membranes in all 4 animals ($12.7 \pm 4.9\%$ reduction, $P < 0.05$ by analysis of variance), when the FIL- treated glands ($75,922 \pm 13,574 \text{ cpm/mg protein}$) were compared to the glands treated with carrier alone ($85,590 \pm 12,314 \text{ cpm/mg protein}$; Figure 4.5). Insufficient availability of membrane prevented determination of IGF-I binding in one pair of glands. There was no correlation between either IGF-I and oPrl binding ($r^2 = 0.151$, d.f. = 10), or between the FIL-induced reductions in IGF-I and oPrl binding ($r^2 = 0.006$, d.f. = 10; results not shown,).

IGF-I binding to both stripped and non-stripped mammary membranes was not affected by either body side or gland position when assessed by analysis of variance ($P > 0.05$, Table 4.1).

Figure 4.4. Scatchard (1949) analysis of [¹²⁵I]-oPrI binding to 4.5 M-MgCl₂ microsomal membranes prepared from lactating rabbit mammary glands 12 hours after the intraductal injection of FIL (●) or carrier (○) into the gland. The results from two animals are shown. The association constant (K_d) of prolactin binding, calculated from the first six values on each graph, was calculated to be 3.35 ± 0.54 × 10⁹ M⁻¹ from both carrier- and FIL- treated glands. An estimate of 210 ± 1 and 121 ± 27 fmol oPrI bound/mg protein was obtained for carrier- and FIL- treated glands respectively.

Animal 1



Animal 2

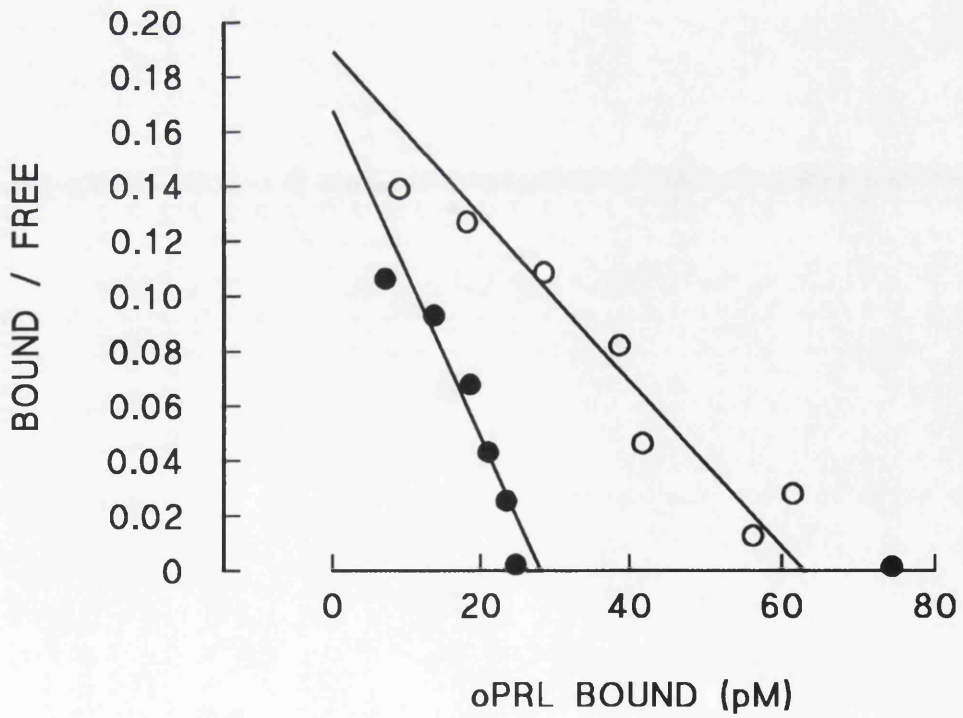


Figure 4.4.

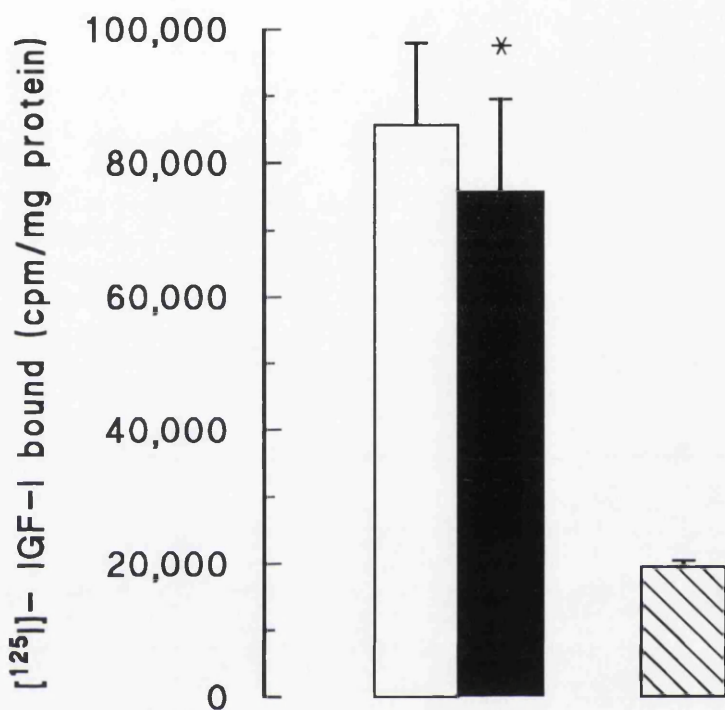


Figure 4.5. Specific [¹²⁵I]-IGF-I binding to 4.5 M-MgCl₂ stripped microsomal membranes prepared from lactating rabbit mammary gland 12 hours after the intraductal injection of FIL (solid bar) or carrier (open bar) into the gland. The specific binding of [¹²⁵I]-IGF-1 to non-stripped microsomal membranes prepared from the liver of a mid-pregnant rabbit (hatched bar) is shown for comparison. Values are means + SEM. (* *P* < 0.05, when compared by analysis of variance with the values for carrier-treated glands).

4.3.3 Direct effect of milk fractions on prolactin binding

Specific binding of prolactin to mammary membranes in the radio-receptor assay was not affected by presence of the 10-30 kDa goat whey fraction (Figure 4.6). FIL increased specific prolactin binding to the mammary membranes by $68 \pm 28\%$ (mean \pm half the range; $n=2$), with there being no specific binding of prolactin by FIL in the absence of membranes.

4.3.4 Mammary gland weight

The weight of the 10-30 kDa goat whey fraction treated mammary glands, 7 and 12 hours after treatment, was 113.0 ± 4.2 and $110.2 \pm 16.8\%$ respectively of that for the carrier-treated (control) glands ($P>0.05$ by analysis of variance). There was also no significant effect of treatment with FIL on mammary gland weight ($21.7 \pm 5.9\%$ increase, $P>0.05$ by analysis of variance).

4.4 DISCUSSION

In order to determine the mechanism by which milk accumulation affects mammary prolactin receptor number (Chapter 3), FIL-containing goat milk fractions were introduced, by intraductal injection, into lactating rabbit mammary glands. The local control of milk secretion is thought to be mediated by FIL (Addey *et al.*, 1991b). Although it has not been demonstrated that this protein is present in rabbit's milk, FIL does inhibit casein secretion by rabbit mammary tissue *in vitro* (Addey *et al.*, 1991b). Further evidence of a physiological role for FIL in the rabbit comes from the demonstration that the 10-30 kDa goat whey fraction, of which FIL is a constituent, reduces milk accumulation and lactogenic enzyme activity *in vivo* (Wilde *et al.*, 1987a; Wilde *et al.*, 1988b),

In the study reported here, the 10-30 kDa goat whey fraction reduced prolactin binding significantly in lactating rabbit mammary tissue. This reduction in prolactin binding was a consequence of the actions of FIL. The effects of FIL on prolactin binding were mediated entirely through changes in receptor number, for there was no effect on prolactin receptor affinity and no direct inhibition of prolactin binding to its receptor. The estimate for the receptor association constant ($K_a=3.35 \pm 0.54 \times 10^9 \text{ M}^{-1}$) was similar to that obtained in Chapter 3 ($K_a=1.89 \pm 0.38 \times 10^9 \text{ M}^{-1}$). Other work also indicates that effects on mammary prolactin binding are a consequence of

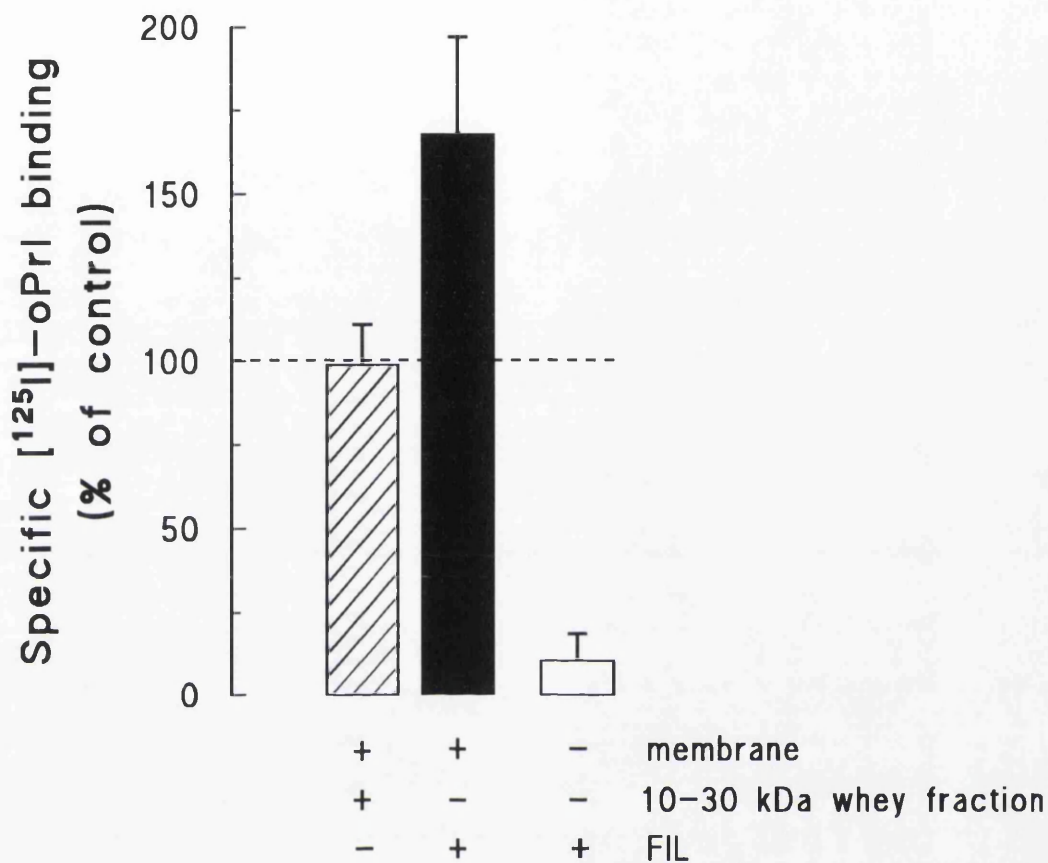


Figure 4.6. Specific binding of [¹²⁵I]-oPrl incubated in the presence (hatched or solid bars) or absence (open bar) of 4.5 M-MgCl₂ stripped microsomal membranes prepared from suckled lactating rabbit mammary glands. The 10-30 kDa goat whey fraction (hatched bar) or FIL (solid and open bars) were also present in the radio-receptor assay. Results are expressed as a percentage of specific [¹²⁵I]-oPrl binding to membranes incubated in the absence of additional whey fractions. Values are means + half the range.

changes in receptor number, with the affinity of the receptor remaining constant (Chapter 3 and (Djiane *et al.*, 1977; Jones & Parker, 1983)). The reduction in prolactin binding was not due to an *in vitro* effect of the whey fraction or FIL being carried over into the radio-receptor assay, for the 10-30 kDa goat whey fraction did not directly affect the binding of prolactin to its receptor. The increased prolactin binding seen with FIL in the radio-receptor assay was the opposite to that seen *in vivo* and cannot be readily explained, but could be due to a perturbation of the membrane environment which is not apparent in the presence of other milk proteins.

That gland weight was not affected following treatment with FIL is in contrast to the findings, over 24 hours, of Wilde *et al* (1987a). However, the study reported here differed from that of Wilde *et al* (1987a) in that no attempt was made to collect the milk escaping from the glands at dissection, for it was necessary to remove and freeze the glands rapidly in order to limit the potential for receptor degradation. This limitation precludes definitive conclusions being drawn from this study on the effects of FIL on milk accumulation.

It is likely, although not certain, that a delay in FIL reaching its site of action was not a significant factor in this study, for in pseudopregnant rabbits, [¹²⁵I]-prolactin can be localised on the alveolar cell within 1 hour of intraductal injection into the gland (Birkinshaw & Falconer, 1972). As a consequence, the reduction in prolactin receptor number 12 hours, but not 7 hours, after the introduction of the 10-30 kDa goat whey fraction into the gland probably represents a cell-mediated delay in FIL affecting prolactin receptor number. This delay contrasts with the rapid changes in receptor number that are seen at suckling (Chapter 3) and may reflect the involvement of the suckling-induced surge in circulating prolactin (McNeilly & Friesen, 1978) in inducing internalisation and down-regulation of the hormone-receptor complex (Djiane *et al.*, 1981a).

The FIL-induced reduction in unoccupied prolactin receptor number, which was measured in non- 4.5 M-MgCl₂ stripped membranes, was in contrast to the slight, but non-significant, increase seen following 24 hours of milk accumulation (Chapter 3). This discrepancy could be explained by differences between the studies in terms of the duration of the gland's exposure to FIL, the concentration of FIL present in the gland and the surge in circulating prolactin at suckling (McNeilly & Friesen,

1978). All these factors may affect the number of receptors which are exposed to circulating prolactin and will in turn influence the number of receptors which remain unoccupied.

The total number of prolactin receptors per cell, which was calculated by expressing the prolactin binding per unit DNA, was lower in the glands treated with FIL, although this was not statistically significant. The reduction in prolactin binding was similar to that obtained when binding was expressed relative to unit protein ($18.6 \pm 12.9\%$ vs. $26.3 \pm 3.4\%$ reduction per unit DNA and protein respectively) and the lack of statistical significance may simply reflect the reduced precision that results from expression of binding with respect to DNA. The estimate of DNA content related to the original mammary tissue and variations in the efficiency of membrane preparation, there being several steps where membrane loss could occur, would affect the resultant estimate of prolactin binding per unit DNA. It was for this reason that prolactin binding was expressed with respect to membrane protein content, with this value representing the end product of the preparation procedure.

This study demonstrated that there was no significant effect of gland position on either mammary prolactin or IGF-I binding, although this does not mean that hormone binding was the same in all glands. However, this does demonstrate that it was statistically correct, in Chapter 3, to compare the effects of milk accumulation on hormone binding by glands at different positions down the body.

The specificity of FIL's effects on mammary hormone receptor number were examined by investigating changes in the gland's binding of IGF-I. The FIL-induced reduction in mammary IGF-I binding suggests that FIL has a general effect on hormone receptors, rather than acting selectively on the receptors of a few galactopoietic hormones. This acute action is in contrast to the differential control seen in goats, where long-term (22 weeks) changes in milking frequency affect prolactin, but not IGF-I, receptor number (Wilde *et al.*, 1990). However, such chronic studies may subject the mammary epithelial cells to differentiative effects other than those acutely-mediated by FIL.

Due to limited availability of IGF-I, no attempt was made to characterise IGF-I binding in this study. The degree of cross-reaction of the IGF-I with the insulin receptor was probably small (Duclos *et al.*, 1989) and there should have been no

cross-reaction with the rabbit mammary gland IGF-2 receptor (Barenton *et al.*, 1987). Specific IGF-I binding in carrier-treated glands was greater than that reported in pregnant glands (Duclos *et al.*, 1989). In contrast, mammary IGF-I receptor number is reduced during lactation in the rat (Lavandero *et al.*, 1990). In the present study, the high level of IGF-I binding may have been due to measurement of total rather than unoccupied IGF-I receptors. Although the effect of 4.5 M-MgCl₂ treatment on the binding of IGF-I to its receptor has not been studied, it can only be assumed that, as for prolactin (Kelly *et al.*, 1979; Van der Gugten *et al.*, 1980), the endogenously-bound IGF-I was stripped from its receptor.

The FIL-induced reduction in hormone receptor number demonstrates that the milk accumulation-induced changes in prolactin binding observed in Chapter 3 were not a consequence of physical distension within the gland. The volume of carrier solution represented a small fraction (5 - 8%) of the milk volume that would be present at the end of the normal suckling interval (Calvert *et al.*, 1987). In addition, if it is assumed that milk accumulation was inhibited in the FIL-treated glands (Wilde *et al.*, 1987a), hormone binding was reduced in those glands which would have had the lower level of physical distension.

The reduction in prolactin receptor number induced by FIL in these experiments and by milk accumulation in Chapter 3, was apparent before there was a significant effect on milk accumulation, but this does not mean that milk secretion had not started to decrease. FIL inhibits constitutive secretion of milk proteins *in vitro* (Rennison *et al.*, 1993), with intracellular casein degradation being stimulated by the 10-30 kDa goat whey fraction (Wilde *et al.*, 1989a). It is tempting to speculate that the FIL-induced reduction in prolactin receptor number was a consequence of the routing of receptors away from the secretory pathway that transports them to the cell-surface, towards a degradative pathway. Alternatively, this receptor down-regulation could be a consequence of an inhibition of protein synthesis, as is observed *in vitro* (Addey *et al.*, 1991b). In both these systems, the reduction in prolactin receptor number would correspond with, or closely follow, the FIL-induced changes in the rate of milk secretion. Such mechanisms would account for the reduction in both IGF-I and prolactin receptor number. The lack of correlation in the reduction of these two receptor types induced by FIL can perhaps be explained by differences in receptor-recycling and turnover, with the IGF-I receptor not being stored in an

intracellular pool or recycled after internalisation (Schalch *et al.*, 1986). It is also possible that FIL regulates milk secretion primarily through modulation of the cell's sensitivity to circulating prolactin, it having been suggested that casein secretion in the rabbit mammary gland is acutely regulated by prolactin (Seddiki & Ollivier-Bousquet, 1991). The mechanism of FIL action is explored further in Chapter 6.

The work performed here does not conclusively prove that FIL is the sole milk protein regulating prolactin receptor number in the lactating mammary gland. However, it is likely that FIL is the only protein in the 10-30 kDa goat whey fraction which significantly down-regulates mammary prolactin receptor number in the rabbit, for the reduction in prolactin binding induced by FIL and the 10-30 kDa goat whey fraction were similar (26% vs. 24% reduction respectively). In addition, if milk protein(s) other than FIL were to affect mammary prolactin receptor number significantly, this effect would be independent of milk secretion, for FIL is the only protein to significantly inhibit milk secretion by rabbit mammary tissue *in vitro* (Addey *et al.*, 1991b). Although it is also possible that non-specific (eg immune) effects arising from the introduction of an exogenous protein into the mammary gland may be responsible for the observed changes in prolactin binding, this effect would again be independent of milk secretion (Wilde *et al.*, 1988b).

4.5 Conclusion

The number of prolactin receptors in the lactating rabbit mammary gland is reduced following the introduction of FIL into the gland. It is likely, that in addition to being the factor responsible for the local control of milk secretion, FIL is also responsible for the local reduction in mammary prolactin receptor number that results from milk accumulation.

CHAPTER FIVE

EFFECTS OF SHORT-TERM CHANGES IN MILKING FREQUENCY ON PROLACTIN BINDING IN THE LACTATING GOAT MAMMARY GLAND

5.1 INTRODUCTION

As milk accumulates in the lactating rabbit mammary gland, there is a significant locally-induced reduction in mammary prolactin receptor number (Chapter 3). This precedes the reduction in the rate of milk accumulation (Calvert *et al.*, 1987). The decrease in prolactin binding is due, at least in part, to the actions of the feedback inhibitor of lactation (FIL), for this protein reduced prolactin and IGF-I receptor number when introduced into the gland via the teat duct (Chapter 4).

In the lactating goat, short-term (4-10 days) changes in milking frequency affect both the mammary gland's sensitivity to circulating prolactin (Knight *et al.*, 1990a) and lactogenic enzyme activities (Wilde *et al.*, 1987b). However, it is not known whether these effects are accompanied by changes in mammary prolactin receptor number, as is observed following long-term (4 weeks) changes in milking frequency (McKinnon *et al.*, 1988).

In order to define the mechanisms controlling milk secretion, mammary differentiation and prolactin receptor number in the lactating goat, changes in prolactin binding and milk yield were investigated following short-term, unilateral alterations in milking frequency.

5.2 EXPERIMENTAL DESIGN AND METHODS

Five British Saanen goats which had kidded between mid-March and mid-April were used in these studies. The animals were housed and fed as described in Chapter 2. The milk yields (by weight) and the time of each milking were recorded.

Study 1

Three goats in week 4 to 9 of their 1st (C8) or 2nd lactation (B1 and B9) were used in this study. The animals, which had previously suckled kids (C8 and B9) or been milked twice daily (B1), were milked once daily in both glands at 08.00 h for 3 days. Subsequently, the right gland only was milked twice daily for 8 days, with the additional milking being at approximately 23.00 h. During this period, the left (control) gland continued to be milked once daily.

Study 2

Two goats (3rd lactation) were milked twice daily in both glands at approximately 08.00 h and 16.00 h, until week 6 to 8 of lactation. For the next 8 days, the right (treated) gland only was milked thrice daily, with the additional milking being at approximately 23.00 h, whilst the left (control) gland continued to be milked twice daily.

At the morning milking on the day of sacrifice, the animals were milked out with oxytocin (0.5 units - intravenous, "Oxytocin-S", Intervet, Cambridge, UK) and immediately sacrificed by exsanguination, following stunning with a captive bolt. Samples of mammary tissue were collected and placed in liquid nitrogen for storage at -20°C. Both 4.5 M-MgCl₂ stripped and non-stripped microsomal membranes were prepared from the tissue and specific binding of [¹²⁵I]-oPrI assessed by radio-receptor assay (Chapter 2). Results were expressed as [¹²⁵I]-oPrI specifically bound (cpm) per mg protein and compared using the Student's paired *t*-test.

Unilateral effects of a change in milking frequency in the test gland were detected by calculation of a relative milk yield quotient (RMYQ) as described by Linzell & Peaker (1971):

$$\text{RMYQ} = (t_2 \cdot c_1) / (t_1 \cdot c_2),$$

where:-
*c*₁ is the yield of the control gland in period 1,
*t*₁ the yield of the test gland in period 1,
*c*₂ the yield of the control gland in period 2 and
*t*₂ the yield of the test gland in period 2.

Period 1 was taken as the 3 days prior to the treatment-period, with period 2 being taken as the final 3 days of the treatment period. An RMYQ value of > 1 indicates that the milk yield of the test gland had increased in period 2 relative to the control gland, whilst an RMYQ of < 1 indicates a decrease in yield relative to the control gland.

5.3 RESULTS

5.3.1 Effects on milk yield

In study 1, the effects of unilateral twice daily (test) milking were compared with once daily (control) milking. Milk yields varied considerably between animals and for this reason, the individual yields are shown in Table 5.1 and Figure 5.1. At the start of the study, when both glands were milked once daily, the milk yield of the test-gland was $129.0 \pm 18.6\%$ (mean \pm SEM, $n=3$) of the control-gland's yield. Unilateral twice daily milking increased the test-gland's milk yield by $91.3 \pm 36.5\%$, with the control-gland's yield rising by $19.0 \pm 9.4\%$. At the end of the study, the yield of the test-gland was $211.9 \pm 53.2\%$ of that for the control-gland (Table 5.1). The unilateral stimulation of milk yield resulting from the increased milking frequency produced a positive RMYQ in all goats (RMYQ = 1.58 ± 0.20), although the response of one goat (B1) was noticeably less than that of the other two (Table 5.1).

The effects of unilateral thrice (test) and twice (control) milking were assessed in study 2, with the individual milk yields being shown in Table 5.1 and Figure 5.2. At the start of the treatment period, the milk yield of the test-gland was 94.5% of the control-gland's yield for both animals. At the end of the treatment period, the yield of the test-gland was 135.5% (A28) and 109.0% (A7) of that gland's pre-treatment yield, whilst the final yield of the control-gland was 123.8% (A28) and 98.5% (A7) of that gland's pre-treatment yield. This local stimulation of milk yield produced a positive RMYQ for both goats (Table 5.1), with unilateral thrice daily milking increasing the milk yield of the test-gland above that recorded for the control-gland (3.4% greater - A28, 4.3% - A7). Milk secretion during the 16 hour period which received the extra milking was higher in the thrice daily milked gland (283 ± 14 g/gland/h, mean \pm half the range) than the twice daily milked gland (186 ± 14 g/gland/h), with secretion during the rest of the day not being affected (132 ± 2 vs. 129 ± 4 g/gland/h, thrice vs. twice).

5.3.2 Characterisation of goat mammary prolactin binding

Specific binding of oPrl to non- 4.5 M-MgCl_2 stripped mammary membranes was linear in the range of 0 - 200 μg membrane protein (Figure 5.3), with 90 μg of stripped or non-stripped membrane being used in subsequent radio-receptor assays. Non-specific binding of [^{125}I]-oPrl to membranes prepared from all glands was 71.8

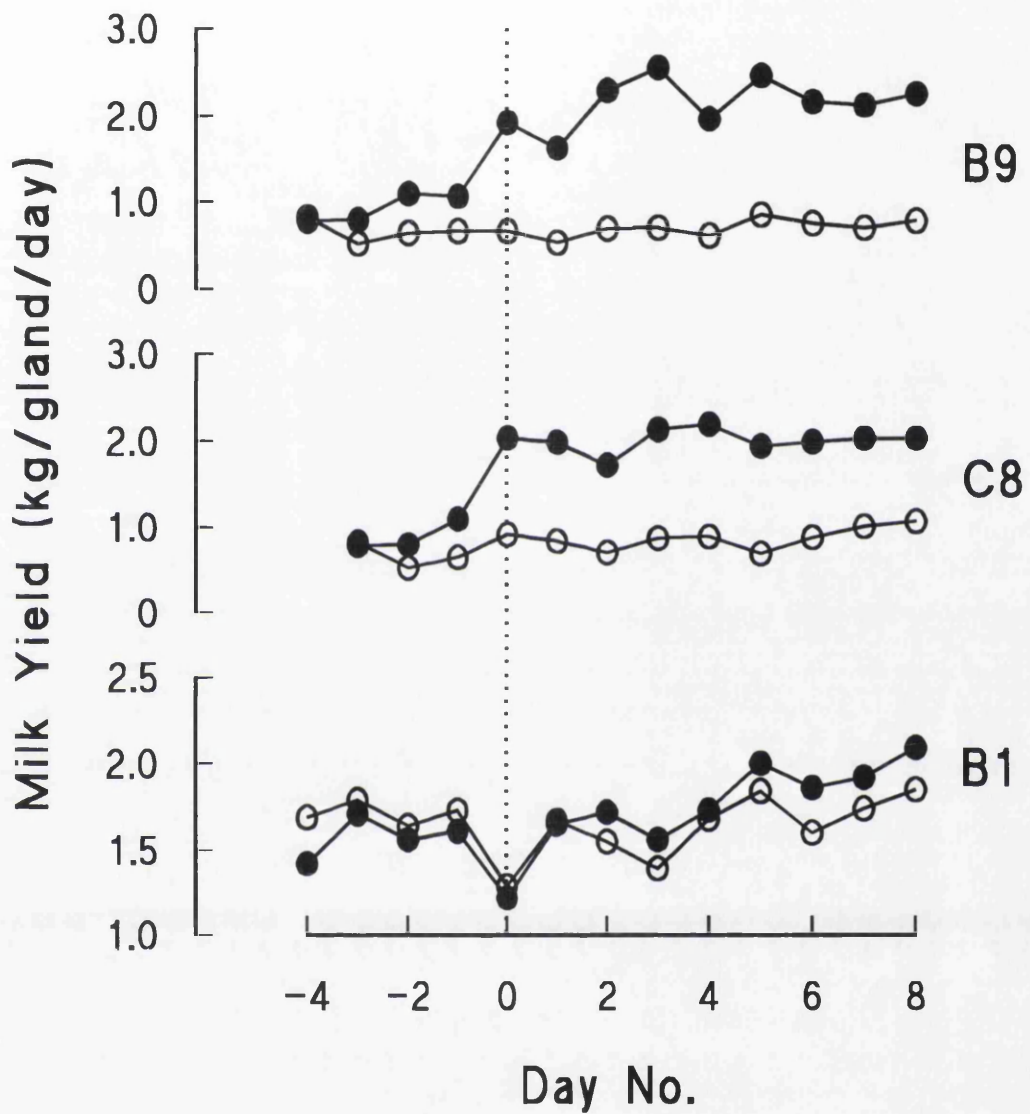


Figure 5.1. Single-gland milk yields of individual goats milked once-daily before being unilaterally milked (from day 0) twice daily (-●-) and once daily (-○-).

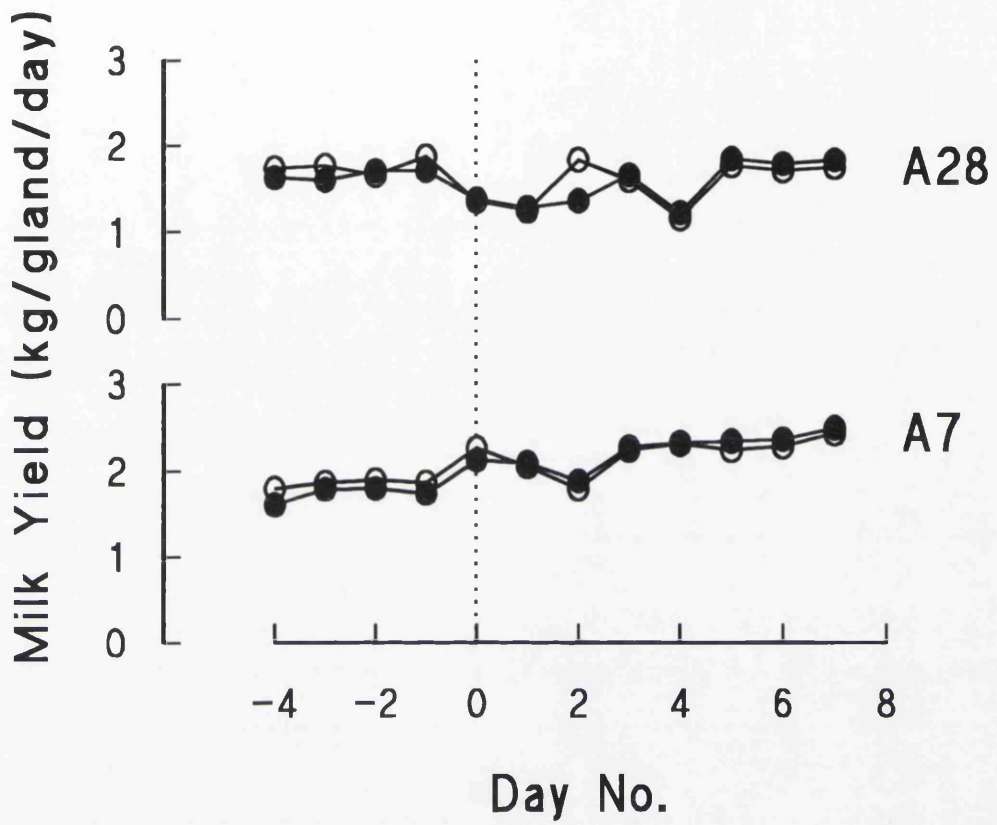


Figure 5.2. Single-gland milk yields of individual goats milked twice-daily before being unilaterally milked (from day 0) thrice daily (-●-) and twice daily (-○-).

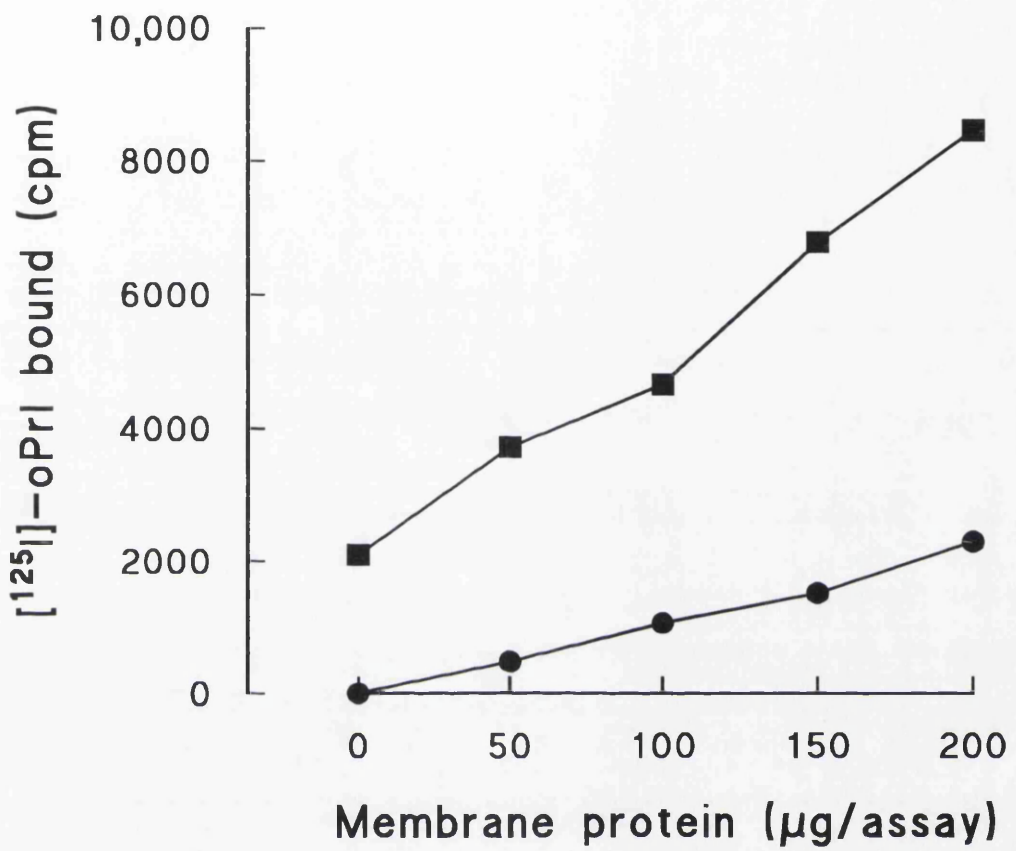


Figure 5.3. Effect of increasing levels of membrane protein in the radio-receptor assay on total (■) and specific (●) binding of $[^{125}\text{I}]\text{-oPrI}$ to non- 4.5 M- MgCl_2 stripped microsomal membranes prepared from lactating goat mammary glands.

Table 5.1. Single-gland milk yield and prolactin binding values for individual goats subject to unilateral alterations in milking frequency. The pre- and post- treatment milk yields are for the 3 days prior to the start of the treatment period and for the final 3 days of the treatment period respectively. RMYQ - Relative milk yield quotient. The mean SEM of specific [¹²⁵I]-oPrl binding to non- 4.5 M-MgCl₂ stripped mammary membranes was ± 1837 cpm/mg protein and ± 3455 cpm/mg protein for stripped membranes.

Specific [¹²⁵I]-oPr1 binding

(cpm/mg protein) (cpm/mg DNA)

Goat	Gland milking frequency (/d)	Milk yield (kg/d)		RMYYQ	Non-stripped			Stripped			
		pre-	post-		stripped	Stripped	Stripped	Stripped	Stripped		
<i>Study 1</i>											
B9	Control - 1x	0.61	0.77	1.83	5,455	37,916	19,298				
		0.97	2.25		8,772	38,275	9,729				
	Test - 2x	0.66	0.86	1.72	9,420	44,953	5,294				
		0.88	1.98		12,981	34,622	7,750				
	C8	1x	1.72	1.73	1.18	7,602	14,427	1,513			
		2x	1.63	1.93		7,346	12,521	2,115			
B1	1x	1.63	1.93	1.18	7,602	14,427	1,513				
	2x	1.63	1.93		7,346	12,521	2,115				
<i>Study 2</i>											
A28	Control - 2x	1.88	2.33	1.09	9,326	43,193	38,167				
		1.78	2.41		11,330	33,046	15,347				
	Test - 3x	1.77	1.74	1.11	11,893	47,303	8,433				
		1.67	1.82		4,003	41,012	3,217				
A7	3x	1.67	1.82	1.11	4,003	41,012	3,217				

Table 5.1.

$\pm 2.6\%$ ($n=10$) of total radioactivity bound for stripped membranes and $80.3 \pm 2.4\%$ ($n=10$) for non-stripped membranes; with $5.7 \pm 0.6\%$ and $2.2 \pm 0.4\%$ respectively of the total radioactivity being bound specifically.

Specifically bound [125 I]-oPrl was displaced by increasing concentrations of unlabelled ovine prolactin (Figure 5.4). Prolactin receptor affinity (K_a), as assessed by Scatchard (1949) analysis of binding data (Chapter 3), was similar in stripped membranes from glands subject to twice and thrice daily milking and was estimated to be $0.83 \pm 0.30 \times 10^9 \text{ M}^{-1}$ (mean \pm half the range, $n=2$), with a representative plot being shown in Figure 5.5.

Milking frequency did not affect the proportion of prolactin receptors which remained unoccupied, with specific prolactin binding by non-stripped membranes representing $29.8 \pm 5.0\%$ ($n=10$) of the binding to stripped membranes.

5.3.3 Effects of milking frequency on mammary prolactin binding

In study 1, specific prolactin binding to stripped membranes was similar in once and twice daily milked mammary glands ($32,432 \pm 9,229$ vs. $28,473 \pm 13,935$ cpm/ mg protein; mean \pm SEM, $n=3$, $P=0.35$; Figure 5.6), with values for individual glands being given in Table 5.1. Prolactin binding to non-stripped membranes was lower in glands milked once rather than twice daily, being most conspicuous in the two animals which showed the largest unilateral milk yield response (B9 & C8). However, this difference was not statistically significant (7493 ± 1146 vs. 9700 ± 1692 cpm/ mg protein, $n=3$, $P=0.22$; Figure 5.6).

In both animals used in study 2, prolactin binding to stripped membranes was lower in thrice daily milked glands than twice daily milked glands ($37,029 \pm 3,983$ vs. $45,248 \pm 2,055$ cpm/mg protein, thrice vs. twice, mean \pm half the range, Table 5.1 and Figure 5.6). Specific [125 I]-oPrl binding to non-stripped membranes was similar in thrice- and twice- milked glands (7667 ± 3664 vs. $10,610 \pm 1284$ cpm/mg protein, thrice vs. twice, Table 5.1 and Figure 5.6).

Prolactin receptor number was similarly unaffected by milking frequency when expressed on a cellular basis, although there was a large variation between animals (Table 5.1). In study 1, specific prolactin binding to stripped membranes prepared from once and twice daily milked glands was 8702 ± 5409 and 6531 ± 2281 cpm/mg DNA (mean \pm SEM, $n=3$, $P>0.05$). In study 2, specific prolactin binding

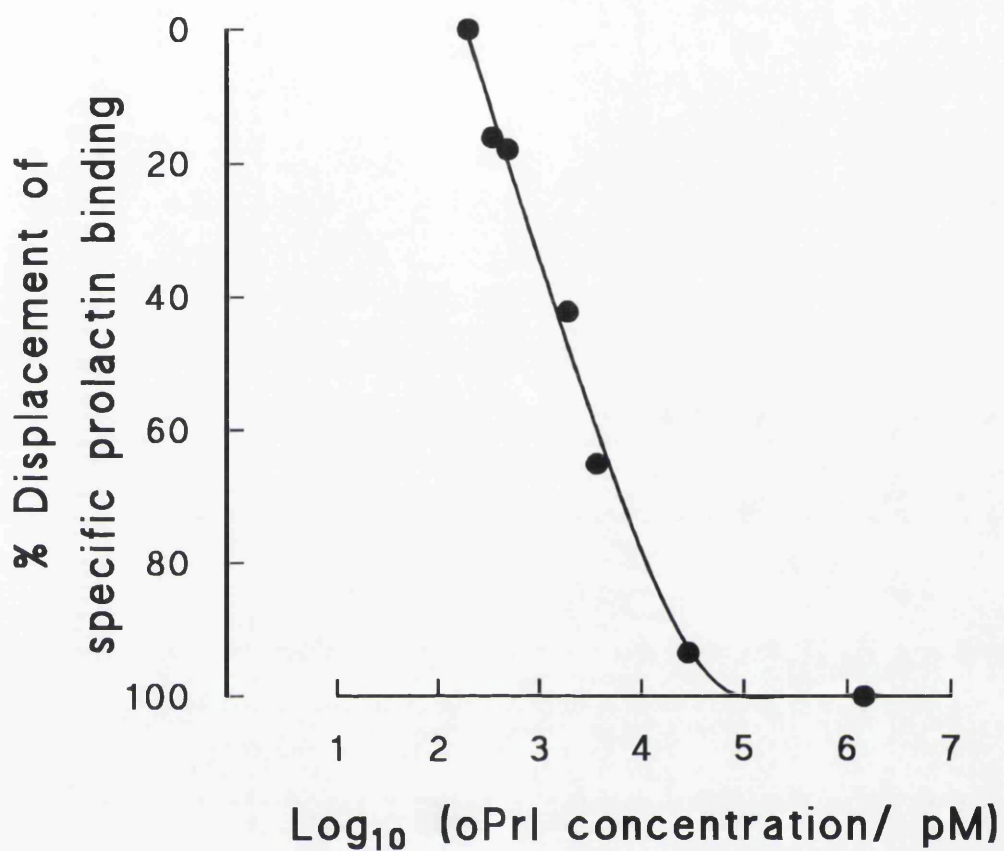


Figure 5.4. Effect of increasing levels of unlabelled oPrl in the radio-receptor assay, on the specific binding of [¹²⁵I]-oPrl to 4.5 M-MgCl₂ stripped microsomal membranes prepared from lactating goat mammary glands.

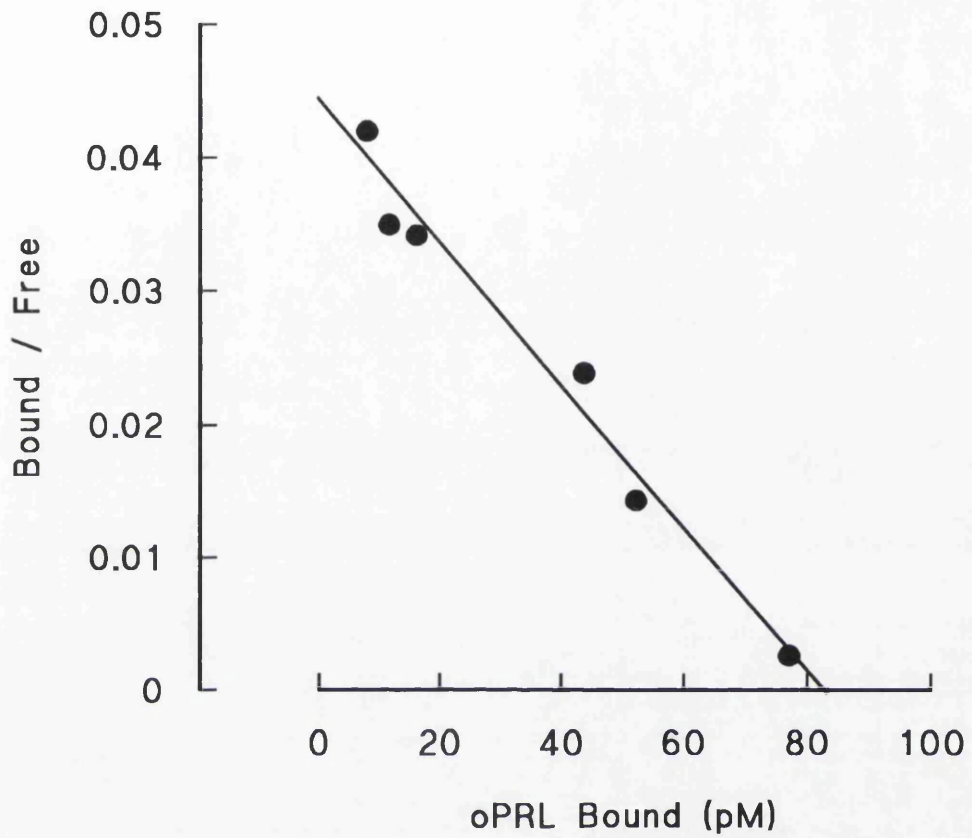


Figure 5.5. Scatchard (1949) analysis of [125 I]-oPrl binding to 4.5 M-MgCl₂ stripped membranes prepared from a lactating goat mammary gland. The association constant (K_a) of the prolactin receptor was calculated to be $0.83 \pm 0.30 \times 10^9 \text{ M}^{-1}$.

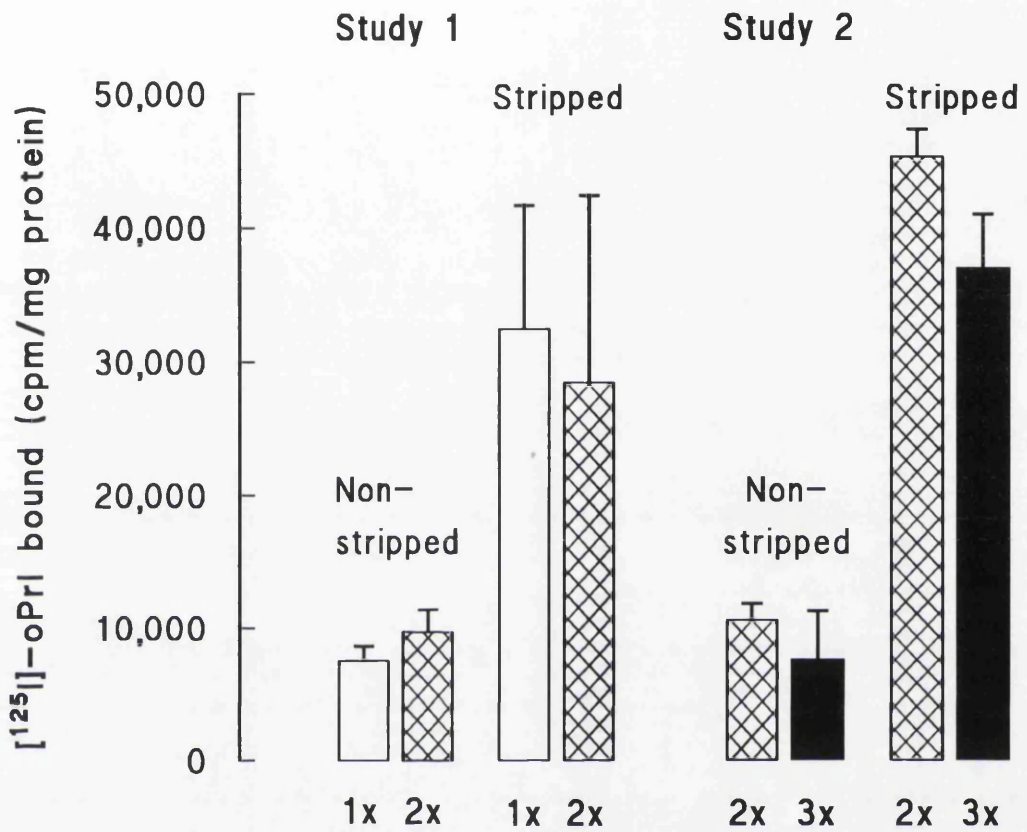


Figure 5.6. Specific [¹²⁵I]-oPrI binding to 4.5 M-MgCl₂ stripped and non-stripped membranes prepared from lactating goat mammary glands subject to unilateral once or twice daily milking in study 1 and unilateral twice and thrice daily milking in study 2. Values are means ± SEM for study 1 and means ± half the range for study 2.

was $23,300 \pm 14,867$ and 9282 ± 6065 cpm/mg DNA (mean \pm half the range, $n=2$) for twice- and thrice- milked glands respectively.

5.4 DISCUSSION

This preliminary study investigated the effects of short-term changes in milking frequency on milk yield and prolactin receptor number in the lactating goat mammary gland.

The binding of ovine prolactin to lactating goat mammary microsomal membranes was through a high-affinity ($K_a = 0.8 \times 10^9 \text{ M}^{-1}$) receptor. The affinity of this receptor was similar to that obtained in lactating rabbit mammary tissue ($K_a = 1.9 \times 10^9 \text{ M}^{-1}$; Chapter 3). Previous studies in ruminants have utilised human growth hormone as ligand (Emane *et al.*, 1986), with the affinity (K_a) of the prolactin receptor estimated to be $0.3 - 0.4 \times 10^9 \text{ M}^{-1}$ (Emane *et al.*, 1986; Akers & Keys, 1984). The occupation of a large proportion (70%) of the prolactin receptors by endogenously-bound hormone contrasts with that observed in the lactating ewe (Emane *et al.*, 1986). The difference in receptor occupancy could reflect the disparate lactational capacities of these two species in addition to methodological differences between the studies, with Emame *et al.* (1986) utilising 2 M-, rather than 4.5 M-MgCl₂ to remove bound hormone from its receptor.

Unilateral more-frequent milking immediately increased the milk yield in the treated-gland, with the control-gland yield increasing to a lesser extent in some, but not all, animals. The effect of an additional daily milking on milk yield was most apparent when applied to once daily milked glands (91% increase) and this response was considerably greater than the 26% increase reported in a previous study (Wilde & Knight, 1990). Positive RMYQ's following thrice/twice daily milking (study 2) indicated that yield increased unilaterally in the thrice daily milked gland of both animals and the size of this response was similar to that reported by Henderson *et al* (1983).

The unilateral increase in milk yield in more-frequently milked glands is a consequence of milk removal and is not due to systemic factors resulting from the additional milking stimulus. The increase in milk secretion is due to removal of a chemical constituent of milk (Henderson & Peaker, 1984), which is thought to be a small milk protein designated FIL (Addey *et al.*, 1991b) and is not due to relief from

the pressure of stored milk (Henderson & Peaker, 1984). The marked variation in responsiveness to more-frequent milking in study 1 probably reflected differences in mammary anatomy, for goats which store a relatively high proportion of their milk in the alveoli (as opposed to the cistern) show a greater response to more-frequent milking (Knight *et al.*, 1989). In addition, the response to more-frequent milking in all animals indicated that milk yield was not being limited by systemic factors (Blatchford & Peaker, 1982).

The local increase in milk yield, stimulated by unilateral more-frequent milking, was not accompanied by an effect on either total or unoccupied mammary prolactin receptor number. This is in contrast to the findings of McKinnon *et al.* (1988), who reported an increase in total mammary prolactin receptor number following 4 weeks of thrice daily milking. However, the increased local response to 4 days of more-frequent milking when the circulating prolactin concentration is low (Knight *et al.*, 1990a) shows that the hormonal sensitivity of the mammary gland can be altered by milking frequency within just a few days, and hence may have been affected in the present study. This would suggest that short-term alterations in milking frequency may affect prolactin sensitivity as a consequence of effects on the sub-cellular localisation of prolactin receptors, rather than through changes in receptor number *per se*.

Changes in receptor sub-cellular localisation, resulting from milk accumulation in the mammary gland, could be a consequence of an inhibition of constitutive protein secretion by FIL (Rennison *et al.*, 1993). This may affect prolactin receptor insertion into the secretory cell surface. It is possible to determine the subcellular localisation of receptors by further purification of the microsomal membranes (Djiane *et al.*, 1981a). However, for an effect on receptor localisation to have been apparent in this study would probably have required tissue sample collection prior to milk removal, for the effects of FIL on milk secretion are rapidly reversed (Wilde *et al.*, 1987a).

It has been suggested that effects on mammary differentiation resulting from alterations in milking frequency or efficiency are a consequence of changes in the gland's sensitivity to circulating prolactin (Wilde *et al.*, 1990). In the present study, casein and α -lactalbumin mRNA levels were not affected by milking frequency (Bryson *et al.*, 1993). In contrast, effects on mammary differentiation can be detected within 2 weeks of unilateral thrice daily milking if relative changes in glands across

an interval of time, rather than absolute differences between glands at a single time, are assessed (Wilde *et al.*, 1987b). It is possible, therefore, that increased milking frequency did induce relative changes in mammary prolactin receptor which, despite the similar initial single gland milk yields, were less than the initial differences between glands in receptor number.

5.5 CONCLUSION

This study demonstrated that prolactin binding and mammary differentiation are not the only factors governing milk yield in the lactating goat mammary gland. The effects of short-term (8 days) unilateral changes in milking frequency on milk yield are not accompanied by effects on prolactin receptor number and are possibly a direct consequence of the regulation of milk synthesis and secretion by local factors, such as FIL.

CHAPTER SIX

EFFECT OF GOAT MILK FRACTIONS ON PROLACTIN RECEPTOR DISTRIBUTION IN ISOLATED LACTATING MOUSE MAMMARY CELLS

6.1 INTRODUCTION

Accumulation of milk within the mammary gland of a lactating rabbit reduces mammary prolactin receptor number (Chapter 3). This decrease in hormone binding is due, at least in part, to the actions of the feedback inhibitor of lactation (FIL, Chapter 4) and precedes the resultant decrease in the rate of milk accumulation (Chapter 3).

In the goat, by contrast, the effects of short-term alterations in milking frequency on milk yield and mammary hormonal sensitivity are independent of mammary prolactin receptor number (Chapter 5). It is possible that this apparent independence could be explained by alterations in receptor sub-cellular localisation (Chapter 5), possibly resulting from the actions of FIL on the gland.

The present study investigated the direct effects of milk protein fractions containing FIL on prolactin receptor number and distribution in the lactating mammary cell. For this purpose, methods were developed for the isolation and culture of mammary cells, by enzymic digestion of mid-lactation mouse mammary gland. This procedure also allowed the acute effects of milk fractions and exogenous prolactin on milk-component synthesis and secretion to be assessed.

6.2 EXPERIMENTAL DESIGN AND METHODS

Isolated cells were prepared by collagenase digestion of the mammary gland of mice at mid-lactation and equilibrated in incubation medium, as described in Chapter 2. Prolactin was not present unless stated.

6.2.1 Effects of prolactin on protein synthesis and secretion

Isolated cells were radio-labelled in culture medium containing 15 $\mu\text{Ci/ml}$ L-[4,5- ^3H]-leucine for up to 3 hours (Chapter 2) in the presence or absence of prolactin (100 ng/ml or 1 $\mu\text{g/ml}$). Incorporation of L-[4,5- ^3H]-leucine into trichloroacetic acid precipitable protein was measured in cells and incubation medium (Chapter 2).

6.2.3 SDS-polyacrylamide gel electrophoresis and fluorography

Cell suspensions were radio-labelled in culture medium containing 100 $\mu\text{Ci/ml}$ L-[^{35}S]-methionine for 2 hours (Chapter 2) in the presence or absence of prolactin (1 $\mu\text{g/ml}$). Proteins in cell and incubation medium samples (equivalent to 40,000 dpm of trichloroacetic acid precipitable protein/lane) were resolved by SDS-polyacrylamide gel electrophoresis (Chapter 2) and their migration compared with molecular weight standards.

6.2.4 Electron microscopy of isolated cells

Cells were fixed for microscopy (Chapter 2) following a 2 hour incubation in the presence or absence of both prolactin (100 ng/ml) and the 10-30 kDa goat whey protein fraction at twice its milk concentration. Electron microscopy was performed by Dr. K.A.K. Hendry as described in Hurley *et al.* (1993).

6.2.5 Development of the [^{125}I]-oPrI binding assay

Binding of [^{125}I]-oPrI to isolated lactating mouse mammary cells was measured as described in Chapter 2. Degradation of [^{125}I]-oPrI during the binding assay was assessed in tracer recovered by centrifugation (Eppendorf, Netheler + Hinz, GmbH.) and stored in liquid nitrogen until required. Trichloroacetic acid precipitation of [^{125}I]-oPrI was performed as described for iodinated peptides in Chapter 2.

6.2.6 Effect of the 10-30 kDa goat whey protein on prolactin receptor localisation

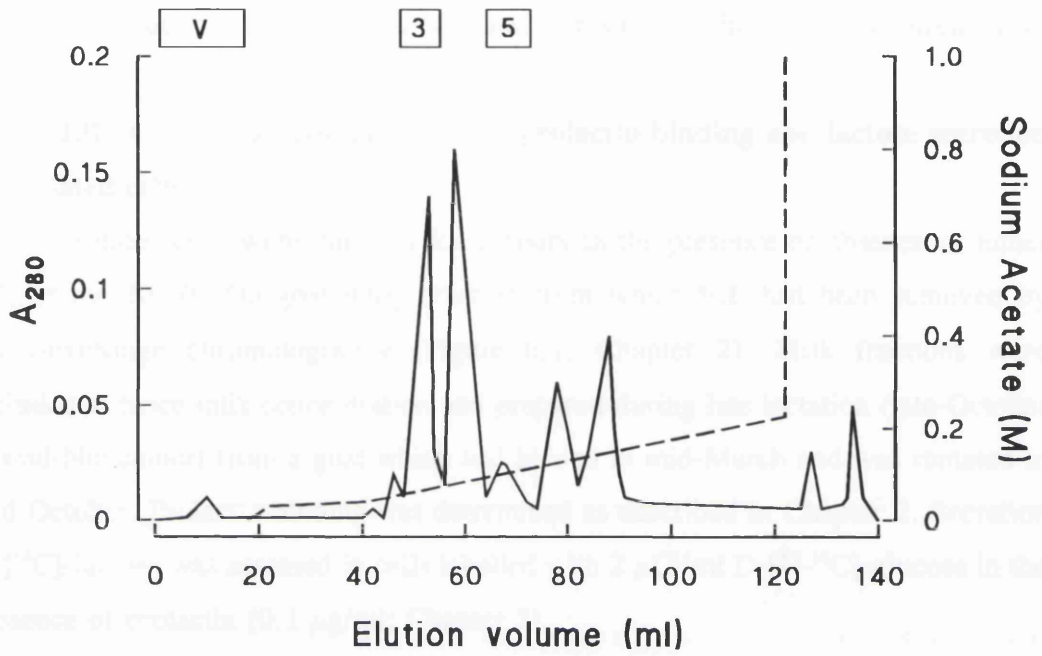
Prolactin binding by intact and digitonin-permeabilised cells was measured following culture for 2 hours in the presence or absence of the 10-30 kDa whey fraction prepared from mid-lactation goat's milk (Chapter 2). This fraction was included at twice milk concentration. Results were expressed as [^{125}I]-oPrI specifically bound (cpm) per μg DNA and analysed using the Student's paired *t*-test.

6.2.7 Effect of FIL and a control milk-protein fraction on prolactin binding and protein secretion by isolated cells

The control protein fraction in these experiments was fraction 5, which is the fifth protein peak resolved when the 10-30 kDa goat whey protein fraction is separated as described for FIL (Figure 6.1, Chapter 2). The effects of FIL and

Figure 6.1. The 10-30 kDa goat whey fraction was resolved by anion exchange chromatography using a Mono Q HR 10/10 column (FPLC System, Pharmacia, Uppsala, Sweden), 10 mM-bis-tris propane pH 7.0 and a 0 - 1.0 M-sodium acetate gradient. FIL was collected as the third resolved fraction (3), whilst fraction 5 was collected as the fifth resolved fraction (5). V - Void volume containing material not bound by the column. Figure a) shows the resolution of the 10-30 kDa fraction prepared from goat's milk collected at mid-lactation. Figure b) shows the resolution of the 10-30 kDa fraction prepared from pregnant goat's milk collected at late-lactation.

a) Mid-lactation



b) Late-lactation

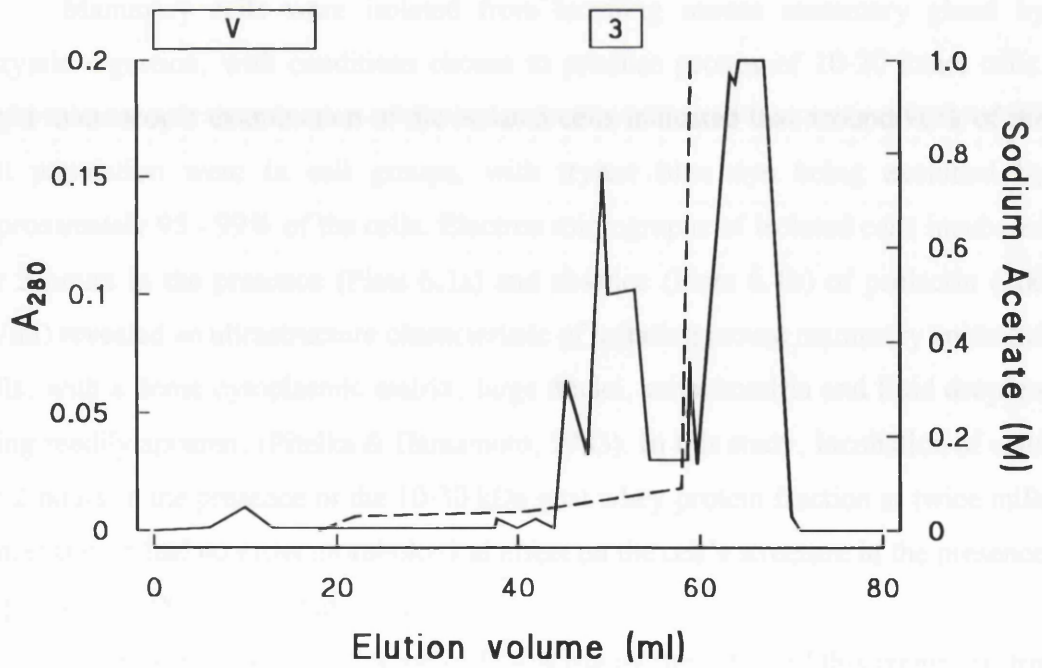


Figure 6.1.

fraction 5 (both at twice their milk concentration) were measured in cells labelled for 2 hours in incubation medium containing 15 $\mu\text{Ci/ml}$ L-[4,5- ^3H]-leucine (Chapter 2). Specific prolactin binding to intact cells (cpm per μg DNA) and secretion of trichloroacetic acid precipitable protein (Chapter 2) were expressed as a percentage of the value obtained for untreated cells and compared by the Student's paired *t*-test.

6.2.8 Effect of FIL in late lactation on prolactin binding and lactose secretion by isolated cells

Isolated cells were cultured for 2 hours in the presence or absence of either FIL or the 10-30 kDa goat whey fraction from which FIL had been removed by anion-exchange chromatography (Figure 6.1, Chapter 2). Milk fractions were included at twice milk concentration and prepared during late lactation (late-October to mid-November) from a goat which had kidded in mid-March and was remated in mid-October. Prolactin binding was determined as described in Chapter 2. Secretion of [^{14}C]-lactose was assessed in cells labelled with 2 $\mu\text{Ci/ml}$ D-[U- ^{14}C]-glucose in the presence of prolactin (0.1 $\mu\text{g/ml}$; Chapter 2).

6.3 RESULTS

6.3.1 Characterisation of the isolated cells

Mammary cells were isolated from lactating mouse mammary gland by enzymic digestion, with conditions chosen to produce groups of 10-20 intact cells. Light microscopic examination of the isolated cells indicated that around 90% of the cell population were in cell groups, with trypan blue dye being excluded by approximately 95 - 99% of the cells. Electron micrographs of isolated cells incubated for 2 hours in the presence (Plate 6.1a) and absence (Plate 6.1b) of prolactin (100 ng/ml) revealed an ultrastructure characteristic of lactating mouse mammary epithelial cells, with a dense cytoplasmic matrix, large nuclei, mitochondria and lipid droplets being readily apparent (Pitelka & Hamamoto, 1983). In this study, incubation of cells for 2 hours in the presence of the 10-30 kDa goat whey protein fraction at twice milk concentration had no gross morphological effect on the cell's structure in the presence of prolactin (100 ng/ml; Plate 6.1c).

There was constant utilisation of D-glucose by the cells and this continued for at least 3 hours (Figure 6.2). There was also a steady utilisation of L-[4,5- ^3H]-leucine, assessed as total cellular ^3H -activity and this was independent of exogenous

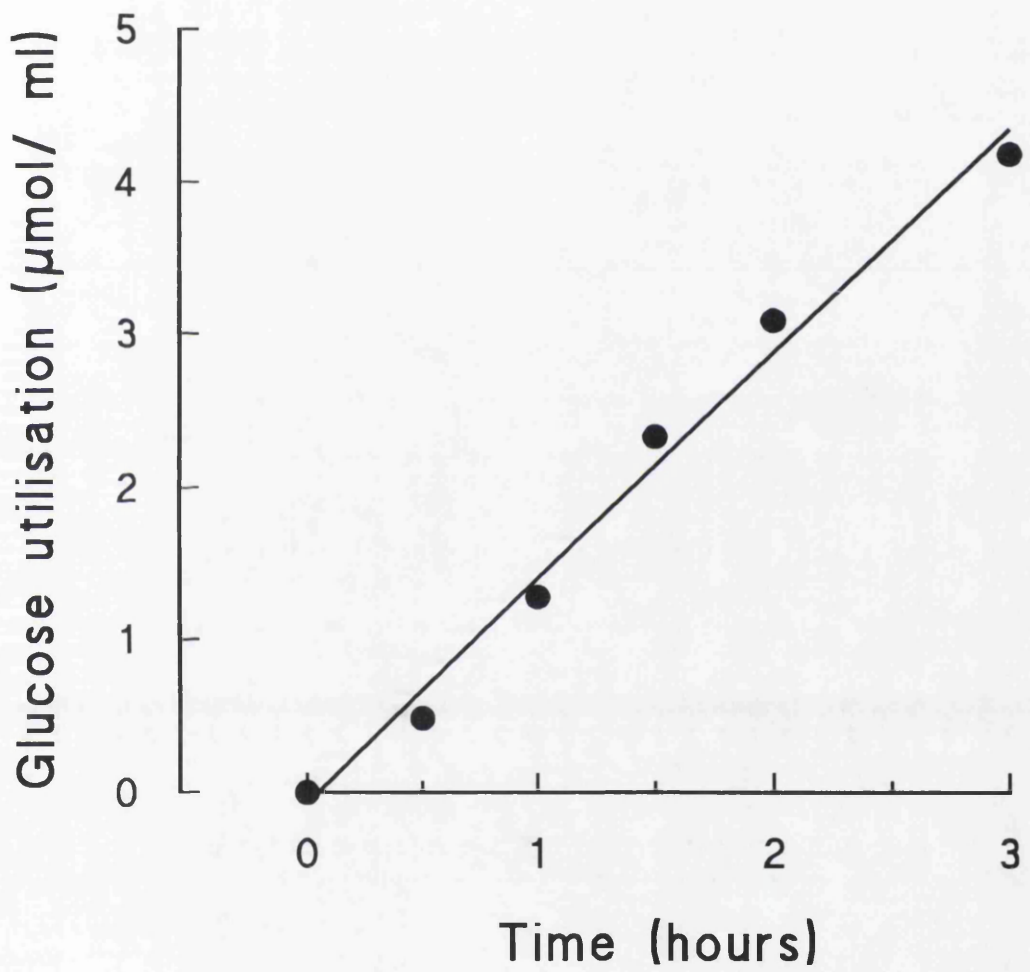
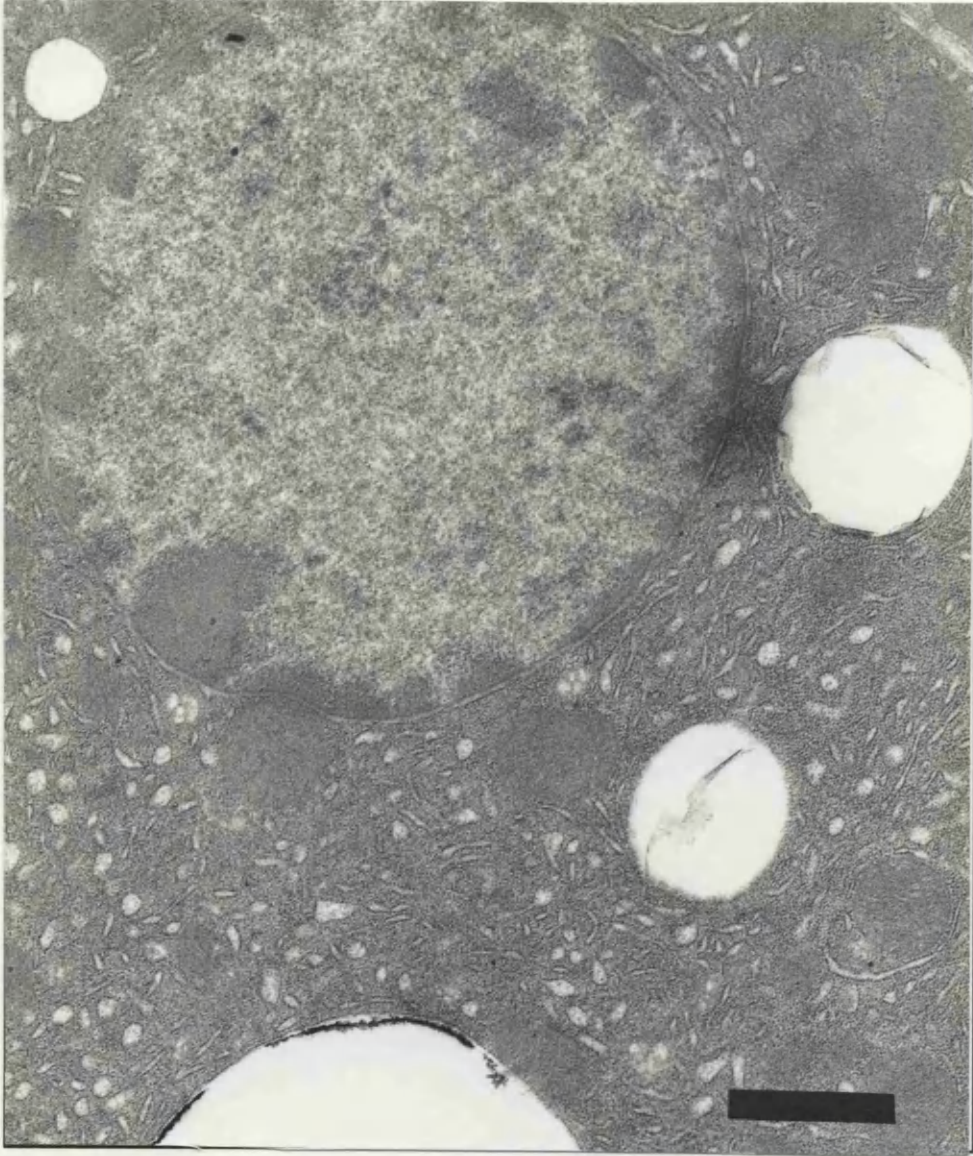


Figure 6.2. Time course of D-glucose utilisation by isolated lactating mouse mammary cells incubated in the absence of prolactin.

a)



b)

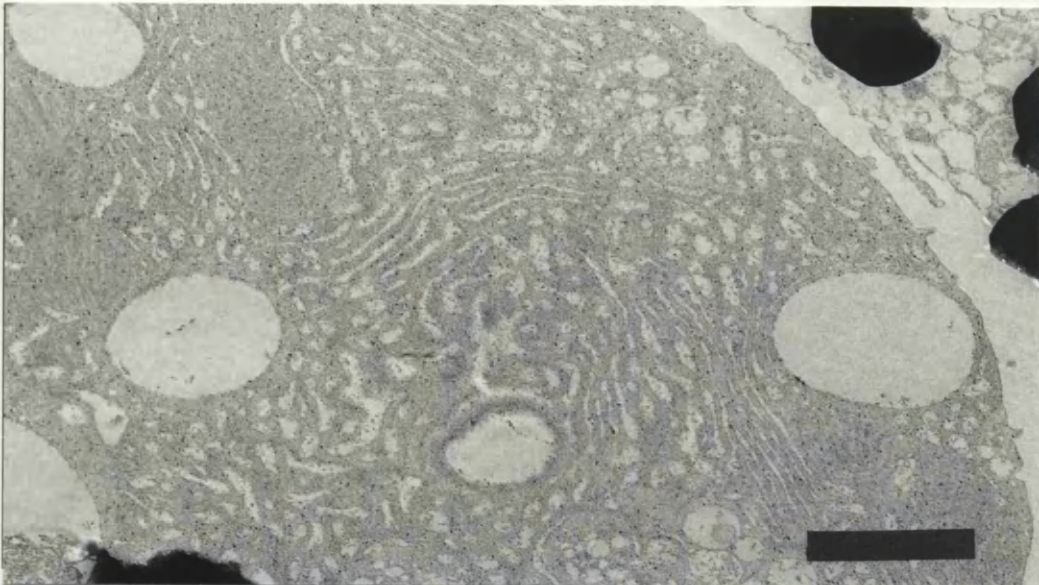
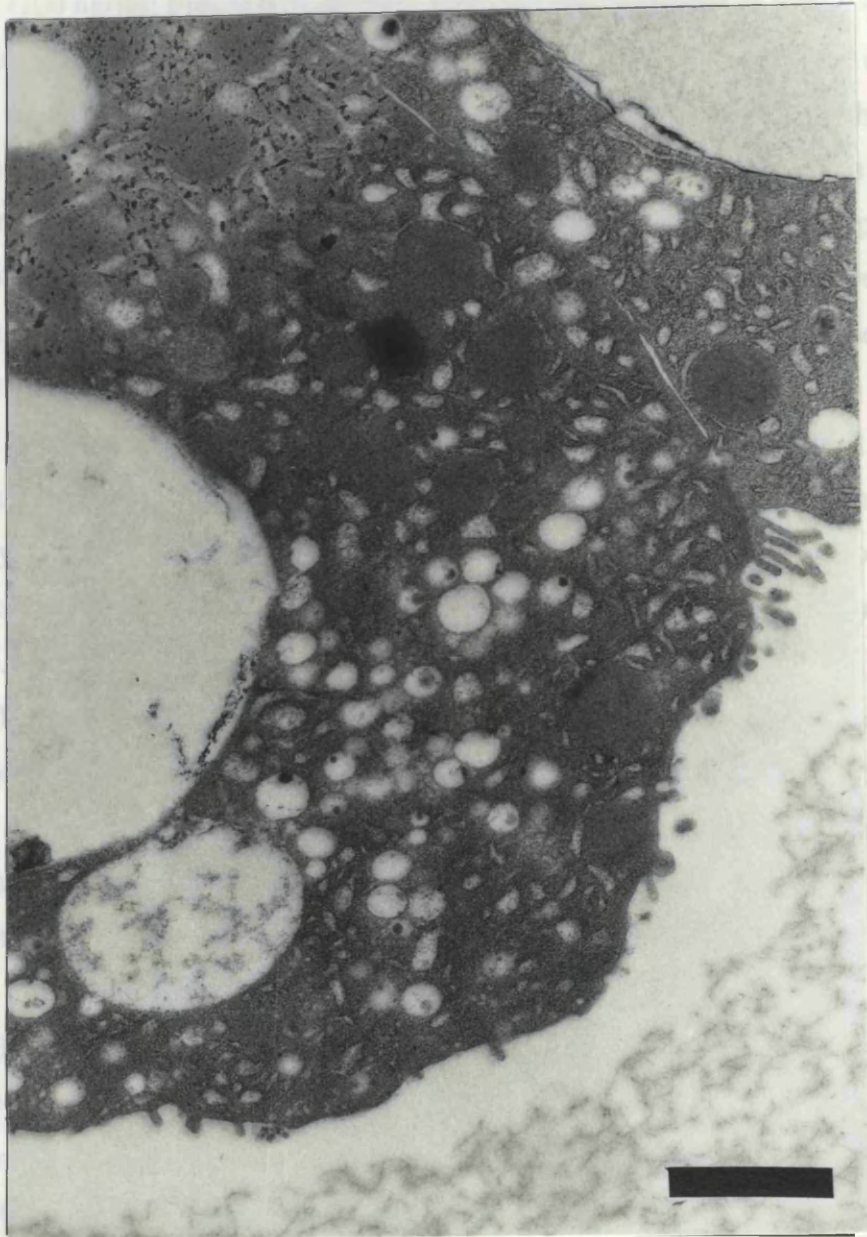


Plate 6.1

c)



presence or absence of prolactin (1 $\mu\text{g/ml}$) (Plates 6.2).

Plate 6.1 Electron micrographs of isolated lactating mouse mammary cells incubated for 2 hours in the a) presence or b) absence of prolactin (100 ng/ml) or c) in the presence of both the 10-30 kDa goat whey protein fraction at twice its milk concentration and prolactin (100 ng/ml). The bar represents 2 μm .

prolactin (100 ng/ml; Figure 6.3). Protein synthesis was measured as total L-[4,5-³H]-leucine incorporated in trichloroacetic acid precipitable protein in both cells and medium. Cells synthesised protein without a detectable lag and a high rate of synthesis continued for at least 3 hours (Figure 6.4). Protein secretion was assessed as total incorporated L-[4,5-³H]-leucine in trichloroacetic acid precipitable protein in the incubation medium. A small, but detectable, amount of protein was secreted after 60 minutes of labelling, with a constant rate of secretion beyond this time (Figure 6.4). After 3 hours, secreted protein accounted for $19.8 \pm 6.6\%$ (mean \pm SEM, n=4) of total synthesised protein. Prolactin in the incubation medium at either 1 $\mu\text{g/ml}$ (n=2, Figure 6.4) or 100 ng/ml (n=2, results not shown) did not affect protein synthesis or protein secretion. Release of ³H-labelled protein was not a result of cell lysis, since the percentage of cells excluding trypan blue was unchanged during this time.

The nature of the proteins synthesised and secreted by the isolated cells was determined by SDS-polyacrylamide gel electrophoresis and fluorography, following labelling of the cells with L-[³⁵S]-methionine for 2 hours (Plate 6.2). The majority of label was incorporated into a limited number of proteins, with selective secretion of milk proteins. Despite the presence of bovine serum albumin, which significantly impaired migration of labelled proteins in the medium samples, it was possible to identify the protein bands. In cell pellets, α - and β -caseins (Green & Pastewka, 1976; Rocha *et al.*, 1985) along with either whey acidic protein (Lee *et al.*, 1984) or α -lactalbumin (Green & Pastewka, 1975) can be identified. The proteins synthesised and secreted by L-[³⁵S]-methionine labelled cells were identical in cells incubated in the presence or absence of prolactin (1 $\mu\text{g/ml}$) (Plate 6.2).

6.3.2 Development of the [¹²⁵I]-oPrl binding assay

Complete and non-reversible permeabilisation of isolated cells was achieved with either 0.006% (w/v) saponin or 0.025% (w/v) digitonin (results not shown). [¹²⁵I]-oPrl bound specifically to both intact and permeabilised mammary cells. Specific binding of [¹²⁵I]-oPrl to intact cells, which in these initial experiments were prepared using a digestion mix supplemented with hyaluronidase (0.5 mg/ml), was maximal at 30 - 45 minutes (Figure 6.5). Non-specific binding represented $72 \pm 4\%$ (mean \pm SEM, n=3) of total radioactivity bound at 45 minutes, the reaction time used subsequently. Cell viability remained high (approximately 95%) during the binding

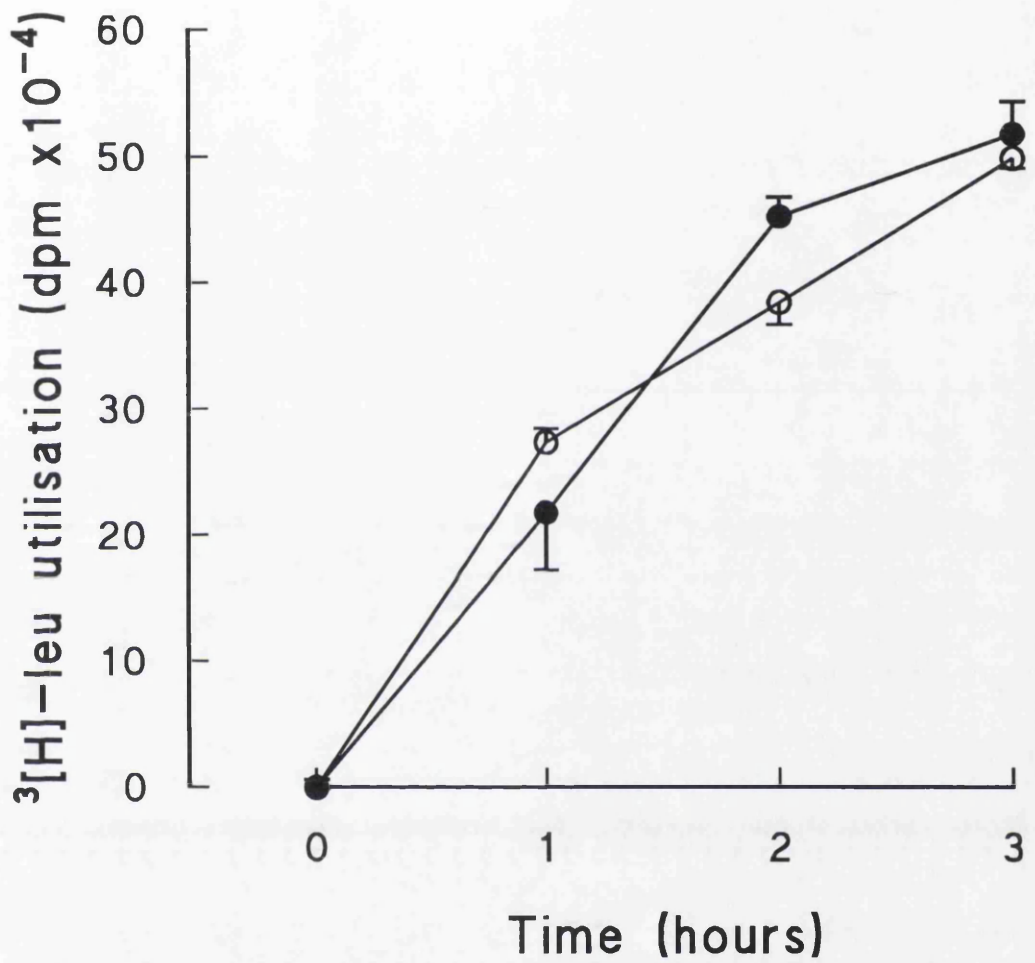


Figure 6.3. Time course of L-[³H]-leucine utilisation, assessed as total cellular ³H-activity, by isolated lactating mouse mammary cells incubated in the presence (-●-) or absence (-○-) of oPrl (100 ng/ml). Values are mean ± SEM for cells incubated in triplicate.

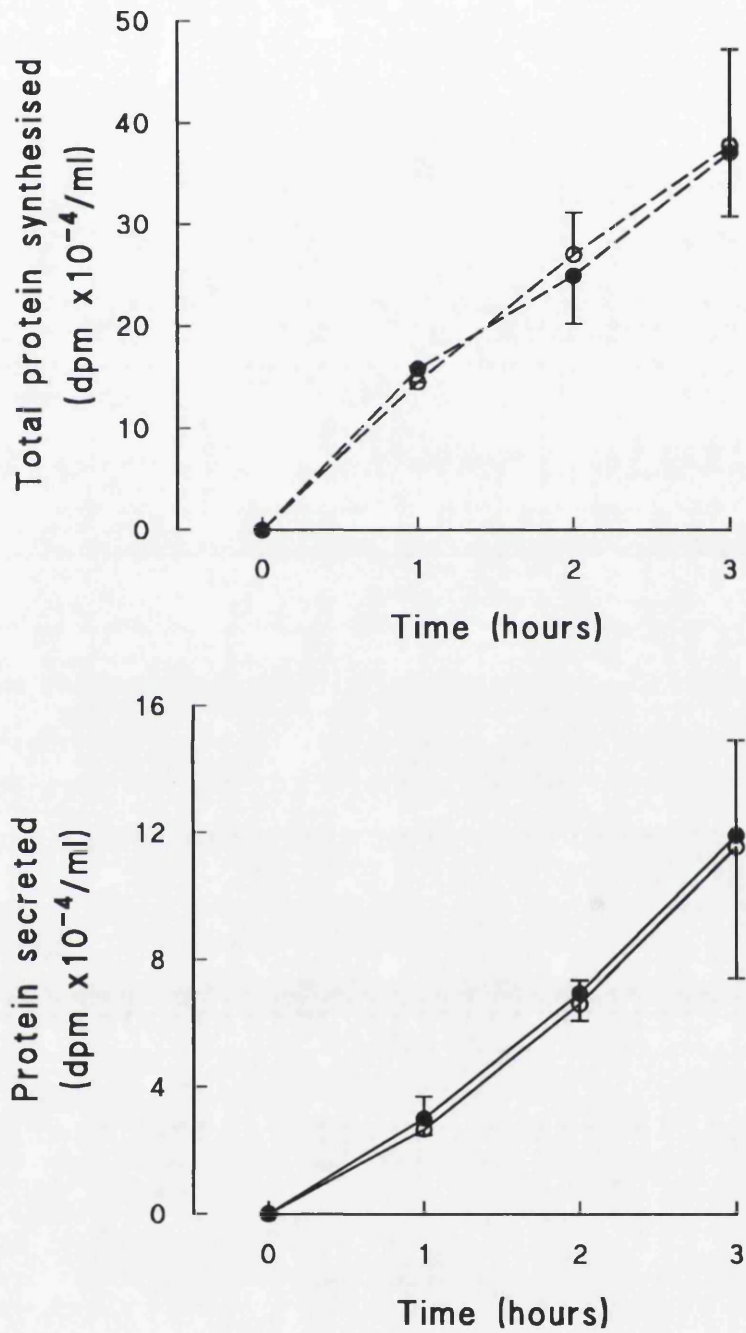
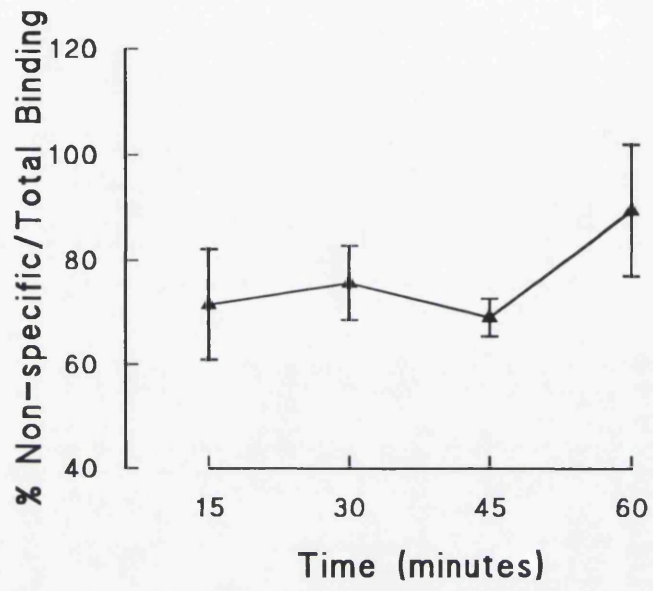


Figure 6.4. Time course of protein synthesis and secretion by isolated lactating mouse mammary cells, incubated with L-[³H]- leucine in the presence (-●-) or absence (-○-) of oPr1 (1 μg/ml). Values are means ± half the range, n=2.

Figure 6.5. Time course of a) non-specific binding of [¹²⁵I]-oPrl, expressed as a percentage of total [¹²⁵I]-oPrl binding and b) specific [¹²⁵I]-oPrl binding, expressed as a percentage of the value obtained for specific binding at 45 minutes, to intact isolated lactating mouse mammary cells. The prolactin binding assay was performed as described in Chapter 2, with the exception that the iodinated peptide was not separated from unincorporated iodine following radio-iodination and that the volumes of the reactants in the binding assay were reduced by a factor of 2. Values are means \pm SEM, n=3.

a)



b)

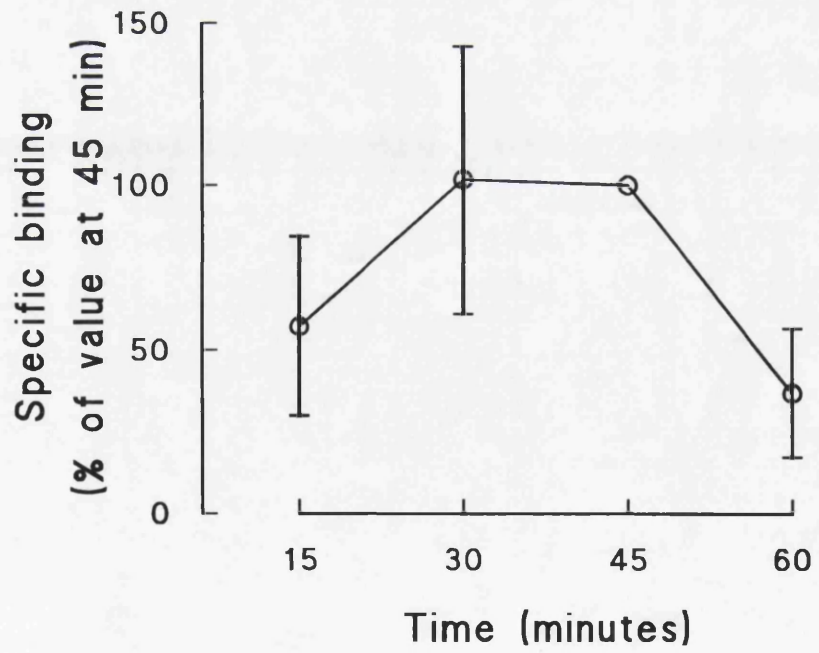
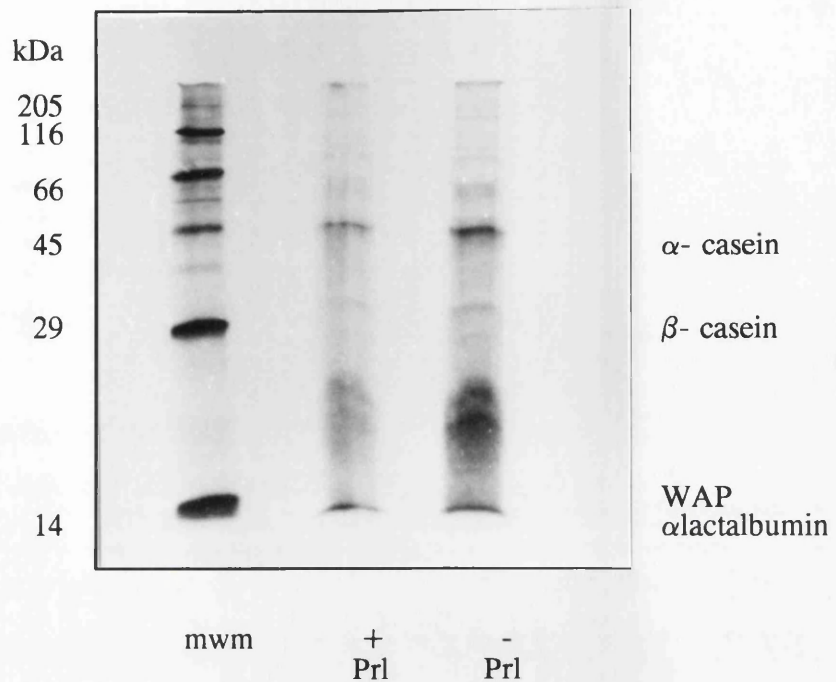


Figure 6.5.

a)



b)

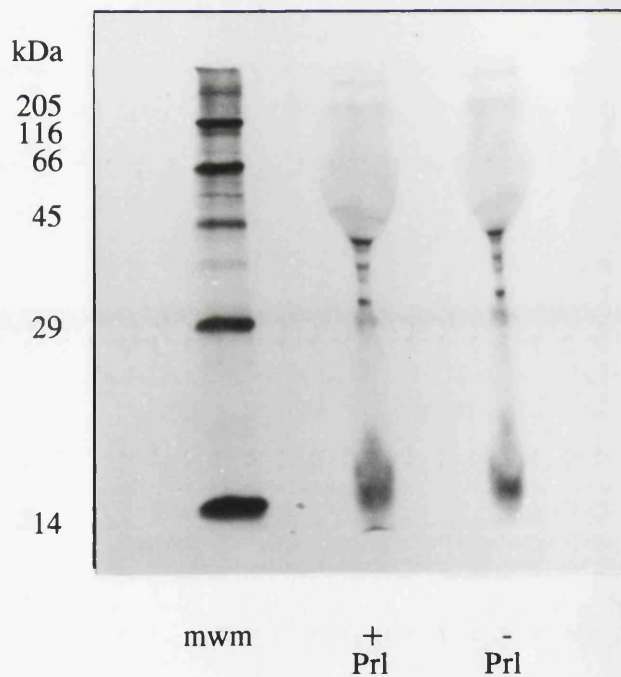


Plate 6.2 SDS-polyacrylamide gel electrophoresis and fluorography of proteins a) synthesised and b) secreted by cells labelled with L-[³⁵S]-methionine for 2 hours in the presence or absence of prolactin (1 μ g/ml). Bands corresponding with molecular weight markers (mwm) and the predominant milk proteins are also shown (WAP - whey acidic protein).

assay, even when the assay was performed 3 hours after preparation of the cells. In permeabilised cells, specific [¹²⁵I]-oPrl binding was maximal at 30 minutes, with 81 ± 8% of total radioactivity being bound non-specifically at 45 minutes (Figure 6.6). Binding of [¹²⁵I]-oPrl to intact cells, when expressed per unit of DNA, was independent of cell number between 8 and 16 μg DNA/assay ($r^2 = 0.17$, d.f. = 17; Figure 6.7), this being equivalent to $0.7 \times 10^6 - 1.4 \times 10^6$ cells/assay when a value of 11.5 pg DNA/cell is used (calculated from Lewin (1957) and Alberts *et al.* (1983)).

Cell-surface specific [¹²⁵I]-oPrl binding, measured in intact cells, was 87 ± 7% (n=4) of that in saponin-permeabilised cells and 88 ± 8% (n=10) of that in digitonin-permeabilised cells.

There was no marked degradation of the [¹²⁵I]-oPrl tracer during the binding assay. In three experiments utilising tracer which had not been separated from unincorporated iodine, 75 ± 1% of the total radioactivity was initially trichloroacetic acid precipitable, with 72 ± 1% of the ligand being precipitated following the binding assay.

Prolactin bound specifically to intact cells was displaced by increasing concentrations of unlabelled prolactin (Figure 6.8). This displacement appeared to be specific for prolactin, in as much as there was no displacement by excess insulin (20 μM). The affinity (K_d) of the prolactin receptor was estimated by Scatchard (1949) analysis to be $8.70 \times 10^7 \text{ M}^{-1}$ with 7.42×10^{-15} moles oPrl bound/μg DNA (Figure 6.9). Although the concentration of unlabelled prolactin required for complete displacement of the ligand was not determined, there was no specific [¹²⁵I]-oPrl binding when prolactin was present at half the concentration (10 μM) routinely used for determining non-specific binding.

6.3.3 Effect of the 10-30 kDa goat whey fraction on prolactin binding

In ten experiments, cell-surface binding of [¹²⁵I]-prolactin to untreated intact cells tended to increase during the 2 hour culture period, although this was not statistically significant (264 ± 32 vs. 333 ± 66 cpm/μg DNA, 0 h vs. 2 h, $P > 0.05$). Incubation of cells in the presence of the 10-30 kDa goat whey fraction reduced cell-surface specific prolactin binding by $24 \pm 8\%$ ($P < 0.01$) when compared with pre-treatment values (Figure 6.10) and by $28 \pm 13\%$ ($P < 0.05$) when compared to untreated cells (206 ± 32 vs. 333 ± 66 cpm/μg DNA, treated vs. untreated).

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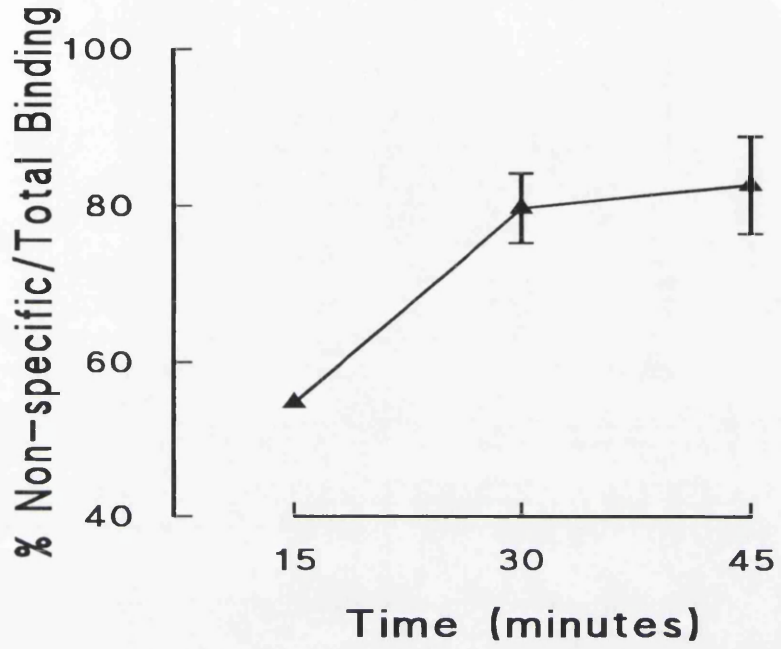
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Figure 6.6. Time course of a) non-specific binding of [¹²⁵I]-oPrl, expressed as a percentage of total [¹²⁵I]-oPrl binding and b) specific [¹²⁵I]-oPrl binding, expressed as a percentage of the value obtained for specific binding at 45 minutes, to saponin-permeabilised isolated lactating mouse mammary cells. The prolactin binding assay was performed as described in Chapter 2, with the exception that the iodinated peptide was not separated from unincorporated iodine following radio-iodination and that the volumes of the reactants in the binding assay were reduced by a factor of 2. Values are means \pm half the range, n=2.

a)



b)

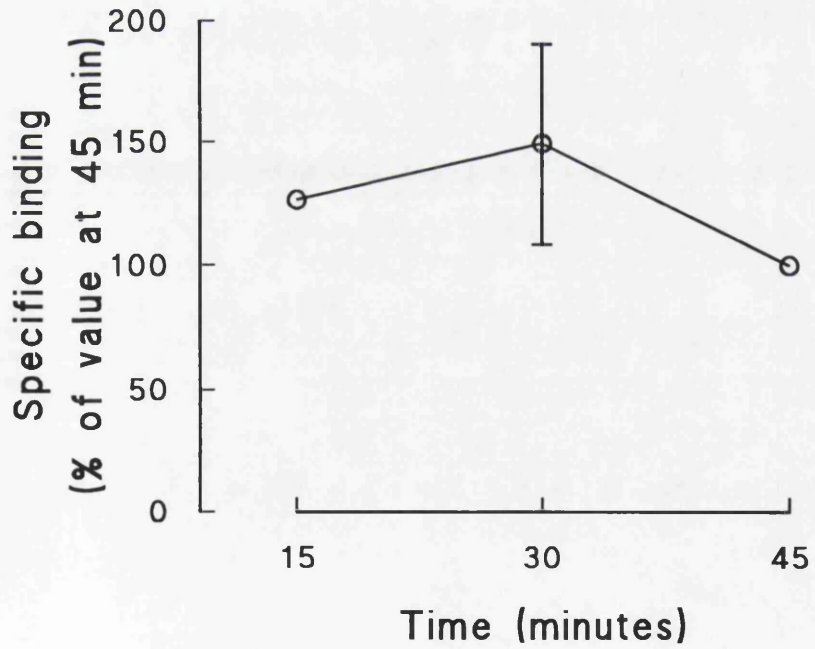


Figure 6.6.

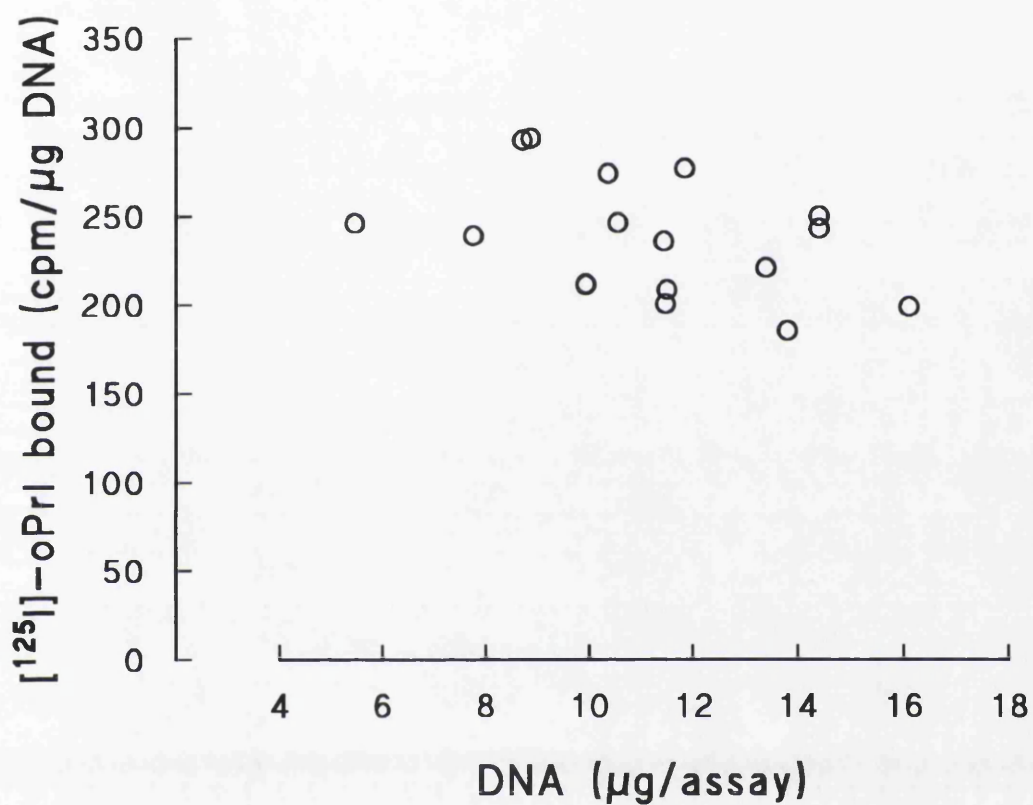


Figure 6.7. Effect of increasing cell number (assessed as μg DNA) in the cell-prolactin binding assay, on specific [¹²⁵I]-oPrl binding to isolated lactating mouse mammary cells when expressed per unit DNA. Correlation coefficient (r^2) = 0.17, d.f. = 17.

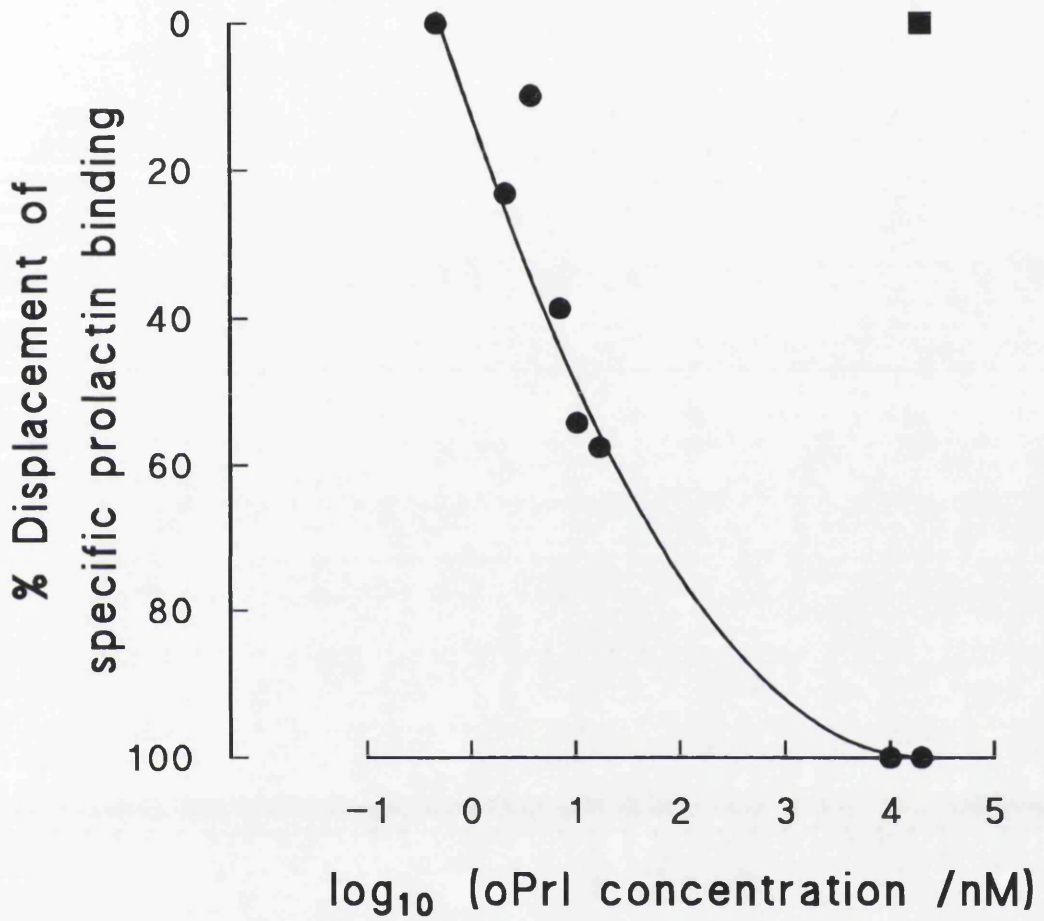


Figure 6.8. Effect of increasing levels of unlabelled oPrl (●) in the cell-binding assay on specific [¹²⁵I]-oPrl binding to isolated lactating mouse mammary epithelial cells. Specific binding of [¹²⁵I]-oPrl in the presence of an excess (20 μM) of unlabelled porcine insulin is also shown (■).

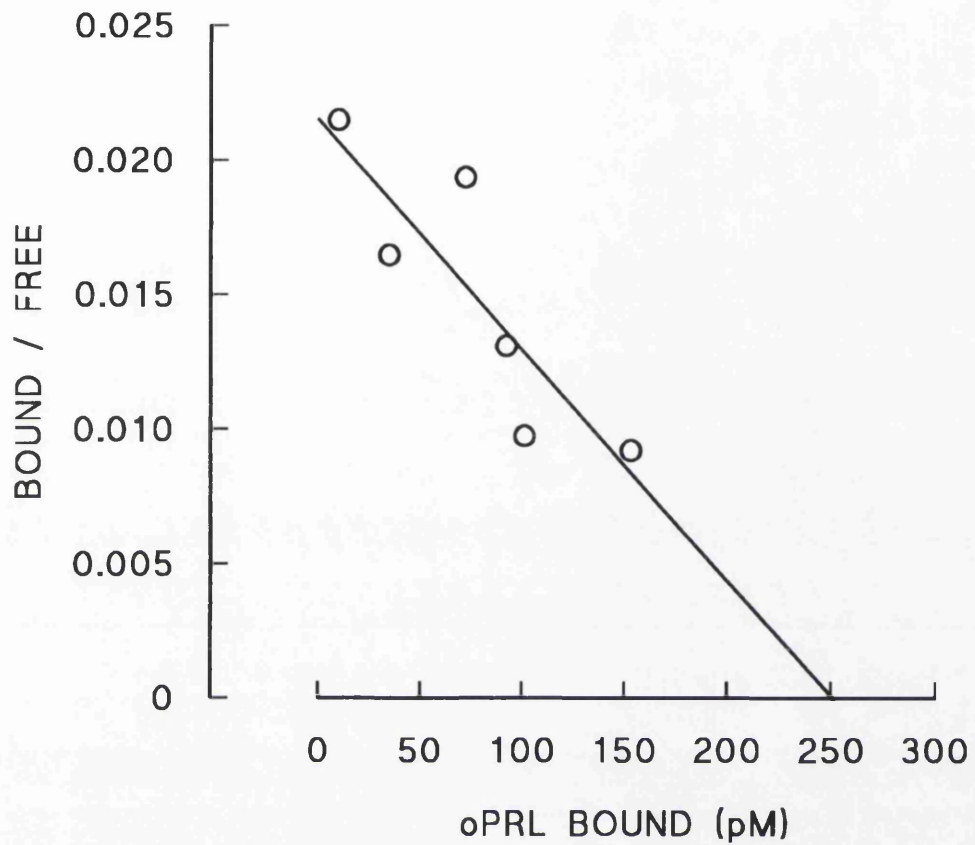
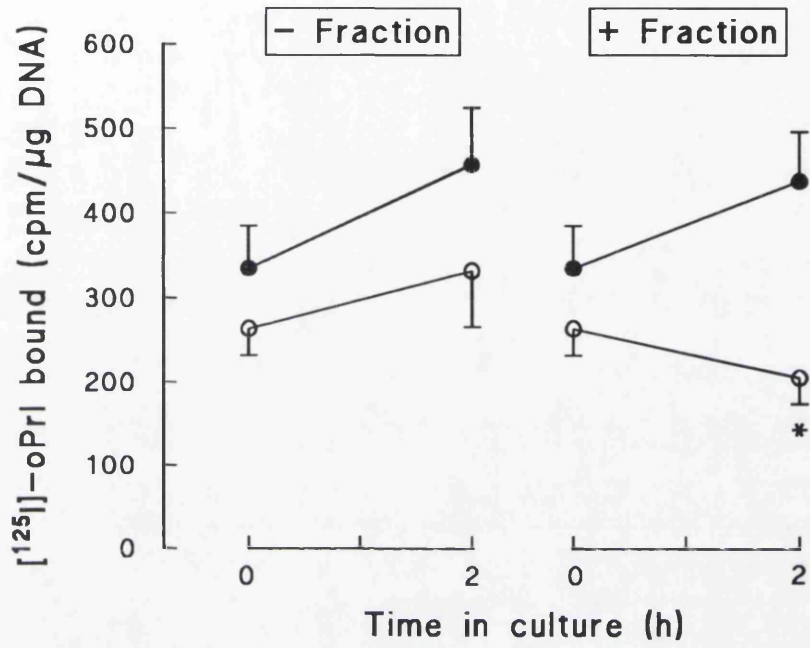


Figure 6.9. Scatchard (1949) analysis of [^{125}I]-oPrl binding to isolated lactating mouse mammary cells. The association constant (K_a) of the prolactin receptor was calculated to be $8.7 \times 10^7 \text{ M}^{-1}$.

Figure 6.10. Effect of culture for 2 hours in the presence or absence of the 10-30 kDa goat whey fraction on **a)** specific [¹²⁵I]- oPrl binding to intact (○) and permeabilised (●) lactating mouse mammary cells and **b)** cell-surface prolactin binding expressed as a percentage of prolactin binding by permeabilised cells. Values are means ± SEM, n=10. (* - *P* < 0.05, when compared to value obtained for cells cultured in the absence of the 10-30 kDa goat whey fraction; ** - *P* < 0.01, when compared to pre-treatment value).

a)



b)

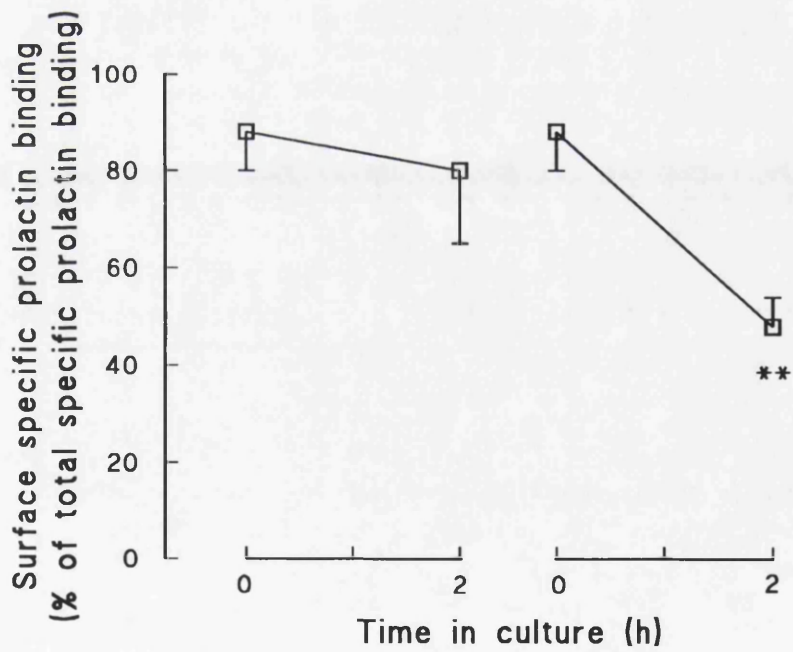


Figure 6.10.

Total specific prolactin binding, assessed in digitonin-permeabilised cells, increased equally in treated (438 ± 58 cpm/ μ g DNA) and untreated-cells (457 ± 67 cpm/ μ g DNA) to $146 \pm 15\%$ ($P < 0.05$) and $145 \pm 14\%$ ($P < 0.05$) of their pre-treatment value (335 ± 25 cpm/ μ g DNA). Consequently, after 2 hours the proportion of prolactin receptors present on the cell-surface was lower ($48 \pm 6\%$ vs. $88 \pm 8\%$, $P < 0.01$) in cells cultured in the presence of the 10-30 kDa goat whey fraction than at the start of culture (Figure 6.10). Although the proportion of receptors on the cell-surface tended to be lower in treated than untreated cells ($48 \pm 6\%$ vs. $80 \pm 15\%$ respectively), this difference was not statistically significant ($P = 0.052$).

6.3.4 Effect of FIL on prolactin binding and protein secretion

Having demonstrated that the 10-30 kDa goat whey fraction affected cell-surface but not total prolactin binding, only surface binding was measured in subsequent studies. Five experiments were performed in this study, although one measurement of proteins synthesis and prolactin binding was not used in subsequent data analysis due to exceptional experimental error.

Cell-surface specific [125 I]-oPrI binding by untreated cells tended to increase during culture, in line with observations in earlier experiments. In contrast, cell-surface prolactin binding was reduced by $24 \pm 5\%$ ($n = 4$, $P < 0.05$) in cells treated with FIL when compared with untreated cells (282 ± 109 vs. 362 ± 122 cpm/ μ g DNA; FIL vs. untreated; Figure 6.11).

The FIL-induced reduction in prolactin binding was associated with a reduced secretion of trichloroacetic acid precipitable protein by cells cultured in the absence of prolactin. Secretion of [3 H]-labelled protein was $40 \pm 7\%$ ($n = 4$, $P < 0.01$) lower in FIL-treated cells than untreated cells (Figure 6.12).

Fraction 5 was used in these experiments as a control milk-protein, since it did not inhibit casein secretion in a tissue culture bioassay (Addey *et al.*, 1991b). However, cell-surface prolactin binding was reduced by $26 \pm 6\%$ (283 ± 109 cpm/ μ g DNA, $P < 0.05$) in cells treated with this fraction when compared to untreated cells (Figure 6.11). This reduction in prolactin binding was similar to that induced by FIL (24% vs. 26% reduction, FIL vs. fraction 5). Secretion of trichloroacetic acid precipitable protein in fraction 5 treated cells was reduced by $25 \pm 5\%$ ($P < 0.05$) when compared to untreated cells (Figure 6.12). Cells incubated with FIL secreted $20 \pm 11\%$ less protein than those incubated with fraction 5 although this difference

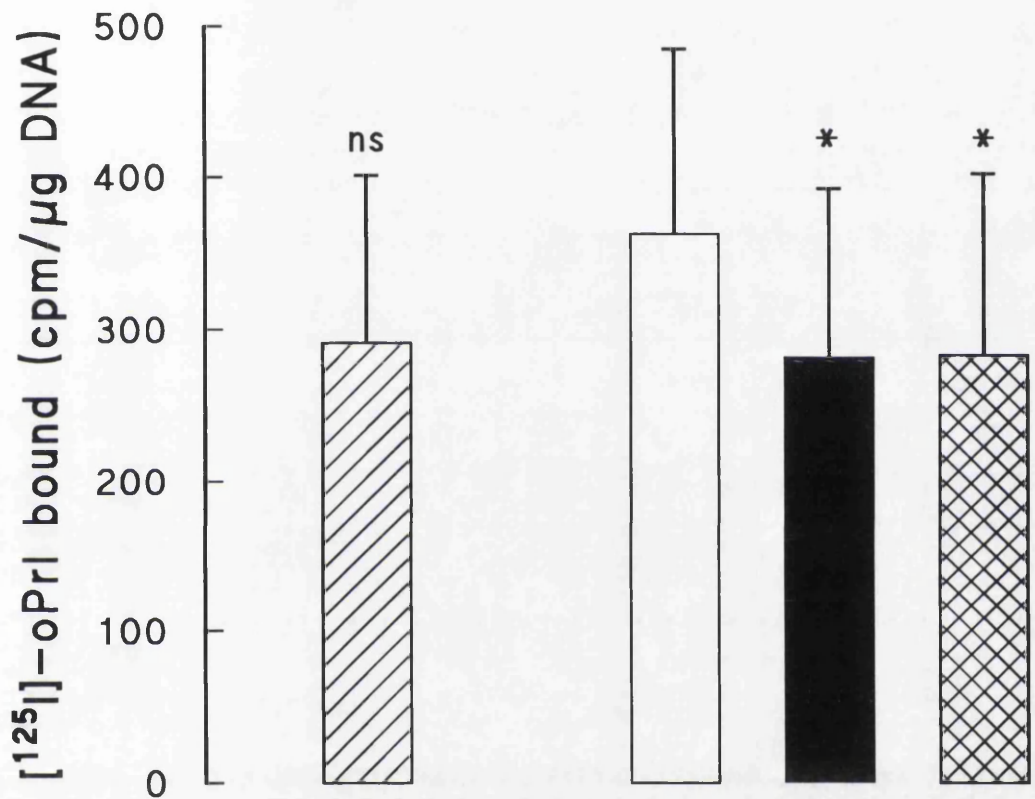


Figure 6.11. Specific [¹²⁵I]-oPrl binding by isolated lactating mouse mammary cells cultured for 2 hours in the absence (open bar) or presence of either FIL (solid bar) or fraction 5 (cross- hatched bar). The pre-treatment value is also shown (hatched bar). Values are means \pm SEM, n=4. (* - $P < 0.05$, ns - not significant, when compared to values obtained for untreated cells).

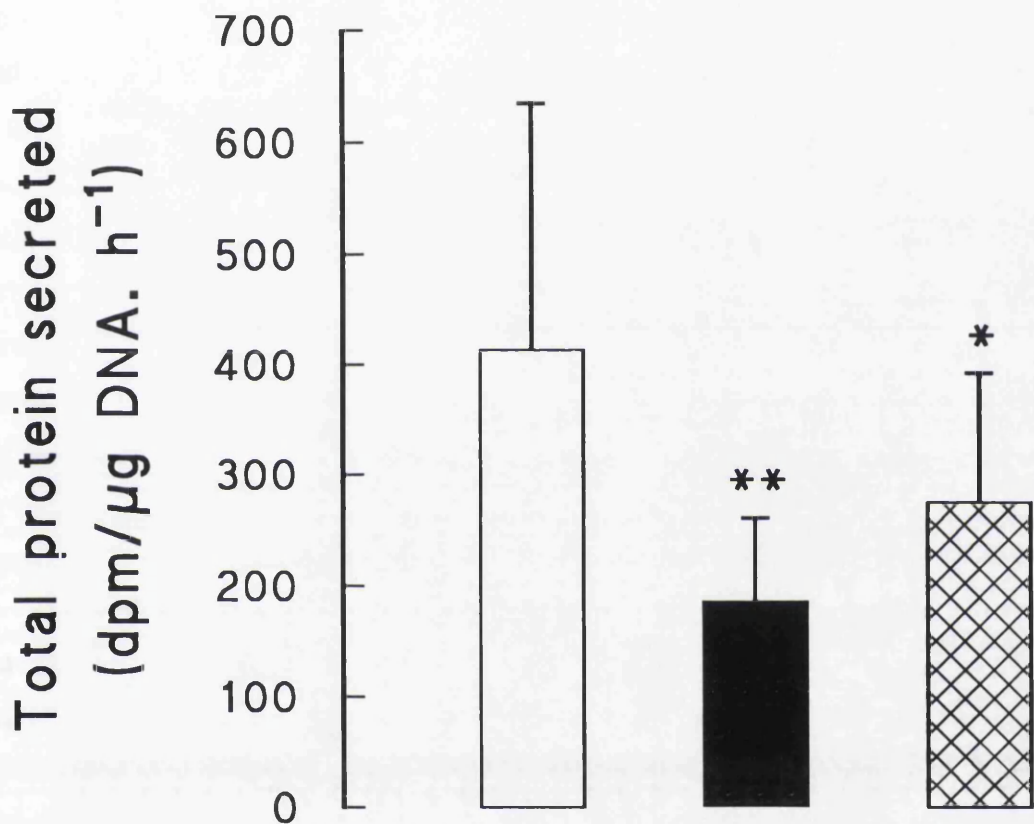


Figure 6.12. Protein secretion by isolated lactating mouse mammary cells cultured for 2 hours in the absence (open bar) or presence of either FIL (solid bar) or fraction 5 (cross-hatched bar). Values are means \pm SEM, $n=4$. (* - $P < 0.05$, ** - $P < 0.01$ when compared to values obtained for untreated cells).

was not statistically significant ($P > 0.05$). Protein secretion tended to be lower in cells binding low levels of prolactin. However, since the relative effects of FIL and fraction 5 on protein secretion and prolactin binding differed there was no correlation between these parameters ($r^2 = 0.02$; d.f.=9, results not shown).

6.3.5 Effect of late-lactation FIL on prolactin binding and lactose secretion

A total of 5 experiments were performed using whey fractions isolated from pregnant goat's milk collected in late lactation. FIL could be resolved at this stage of lactation, although the protein profile differed markedly from that obtained during mid-lactation (Figure 6.1). Specific prolactin binding by cells incubated for 2 hours in the presence of either FIL (191 ± 49 cpm/ μ g DNA) or the 10-30 kDa goat whey fraction minus FIL (167 ± 35 cpm/ μ g DNA) was similar to the value obtained for untreated cells (204 ± 69 cpm/ μ g DNA; Figure 6.13). In three experiments in which lactose secretion was measured, there was no consistent effect of FIL on this parameter (Figure 6.14). Although cells incubated with the 10-30 kDa goat whey fraction minus FIL tended to secrete less lactose than untreated cells, this was not statistically significant ($P > 0.05$; Figure 6.14). Although cells with high levels of prolactin binding tended to secrete more lactose, this correlation was not statistically significant ($r^2 = 0.439$, d.f.=8; Figure 6.15).

6.4 DISCUSSION

6.4.1 Characterisation of isolated cells

Following isolation of lactating mouse mammary cells, leucine and glucose utilisation together with protein synthesis and secretion were constant over the time period in which the effects of milk fractions were investigated. Although a non-metabolisable amino acid, such as α -aminoisobutyric acid, is required to quantify amino acid uptake accurately (Mephram, 1988), changes in amino acid uptake and metabolism should be apparent using a physiological and metabolisable amino acid such as leucine. Similar studies in isolated mouse mammary cells have demonstrated protein secretion consistent with constitutive secretion of proteins immediately following movement through the secretory pathway (Rennison *et al.*, 1993). The proportion of synthesised protein that was secreted in the present study was comparable with other studies, both *in vivo* (Saacke & Heald, 1974) and *in vitro* (Rennison *et al.*, 1993) and appeared to be specific for milk proteins. In addition, the

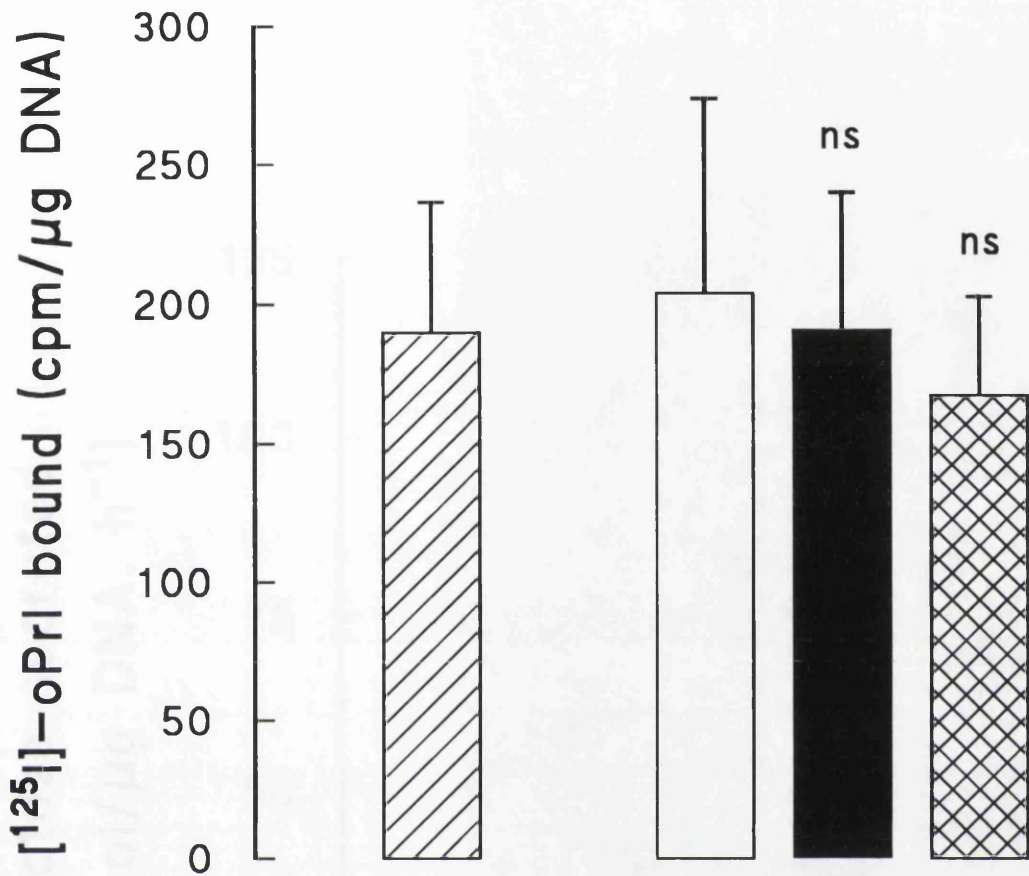


Figure 6.13. Specific [¹²⁵I]-oPrI binding by isolated lactating mouse mammary cells cultured for 2 hours in the absence (open bar) or presence of either FIL (solid bar), or the 10-30 kDa goat whey fraction minus FIL (cross-hatched bar), prepared from goat's milk collected in late-lactation. The pre-treatment value is also shown (hatched bar). Values are means \pm SEM, n=5. (ns - not significant, when compared to values obtained for untreated cells).

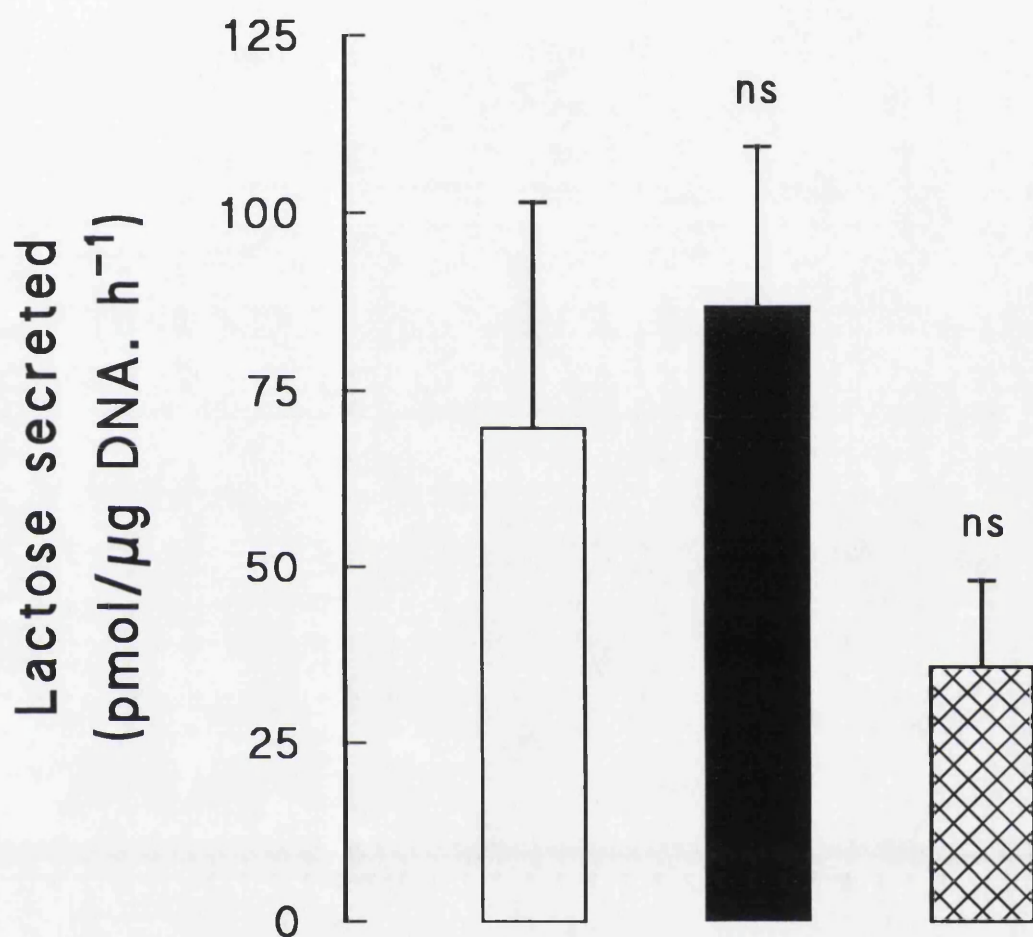


Figure 6.14. Lactose secretion by isolated lactating mouse mammary cells cultured for 2 hours in the absence (open bar) or presence of FIL (solid bar), or the 10-30 kDa goat whey fraction minus FIL (cross-hatched bar), prepared from goat's milk collected in late-lactation. Values are means \pm SEM, $n=3$. (ns - not significant, when compared to values obtained for untreated cells).

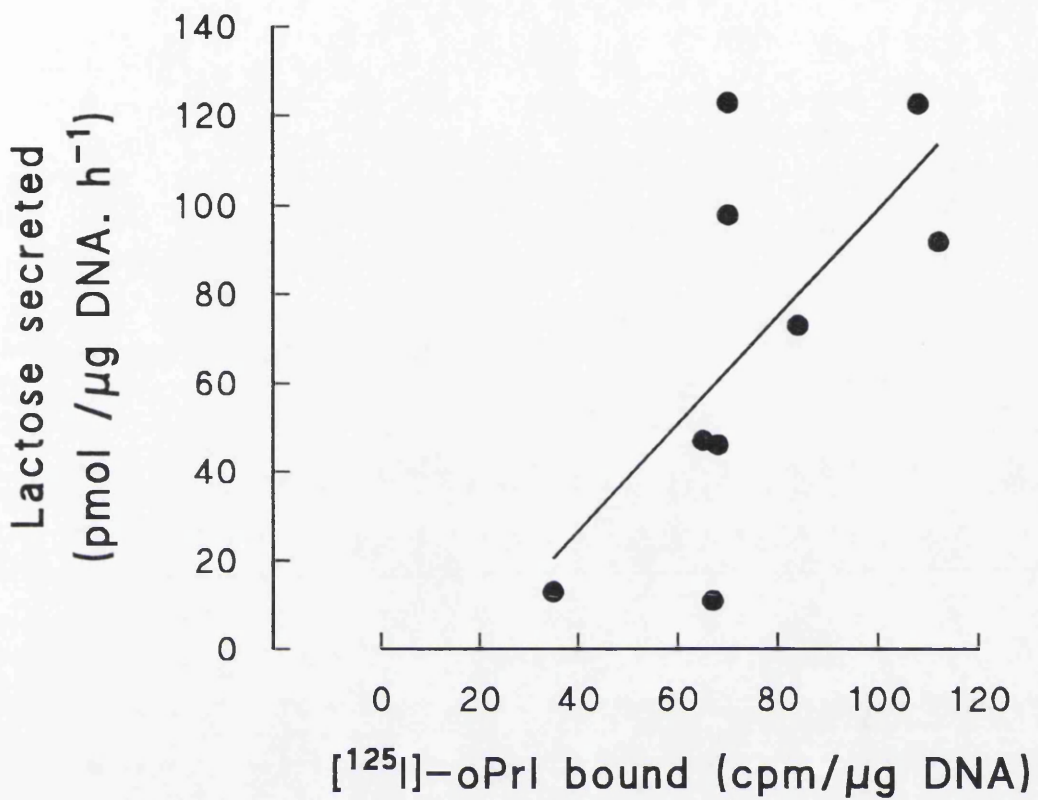


Figure 6.15. Correlation of lactose secretion and specific [¹²⁵I]- oPrI binding by isolated lactating mouse mammary epithelial cells cultured for 2 hours in the presence or absence of either FIL, or the 10-30 kDa goat whey fraction minus FIL, prepared from goat's milk collected in late-lactation. Correlation coefficient (r^2) = 0.439, d.f. = 8.

ultrastructure of the isolated cells bore many of the characteristics of cells *in vivo*, with incubation with the 10-30 kDa goat whey fraction, at twice milk concentration, or in the presence or absence of prolactin having no gross effect on morphology.

Incubation of isolated cells in the presence or absence of prolactin did not affect protein synthesis or secretion during a 3 hour culture period. The concentrations of exogenous prolactin used in these experiments have previously been shown to stimulate maximal α -aminoisobutyric acid uptake in mouse mammary explants (Rillema *et al.*, 1992) and induce cell differentiation in primary mammary cell cultures (Wilde *et al.*, 1991). The lack of a response by the isolated cells to exogenous prolactin could be explained by the carry-through of endogenous prolactin into the incubation medium. However, this is unlikely as a high level of specific [¹²⁵I]-oPrI binding was obtained in the prolactin-binding assays, suggesting that most of the receptors were not occupied by the hormone. It is possible that the lack of response to prolactin in the present experiments was due to preexisting maximal stimulation, rendering the prolactin stimulus that acted on the cells during the culture period superfluous.

The absence of a prolactin effect in this study may be because prolactin acts following a significant lag-period. In rodents, when mammary differentiation is being induced, neither amino acid uptake *in vitro* (Rillema *et al.*, 1992) or milk secretion *in vivo* (Flint *et al.*, 1992) are affected until at least 4 hours after prolactin's administration. With such a time-lag, an effect of prolactin would not have been apparent during the 3 hour incubation period used here. Once prolactin is administered its effects on mammary differentiation are sustained, a single injection of prolactin augmenting milk yield for more than 15 hours *in vivo* (Flint *et al.*, 1992). In the present study, the isolated mouse cells may have retained a highly differentiated state as a consequence of this prolonged action, i.e. as a result of the galactopoietic stimulation of the mammary glands by endogenous hormone prior to the tissue's removal from the animal.

These observations contrast with those in the rabbit, where mammary differentiation can be rapidly reversed (Wilde *et al.*, 1988b), with prolactin having an acute action on both potassium ion uptake (Falconer *et al.*, 1983) and casein secretion (Seddiki & Ollivier-Bousquet, 1991) *in vitro*. The rapidity of prolactin action in the rabbit *in vitro* may be a consequence of effects which act to prevent de-differentiation rather than to stimulate further differentiation through gene transcription.

6.4.2 Characterisation of prolactin binding and the effects of milk fractions

Binding of prolactin to the surface of isolated cells was through a high-affinity binding site which was apparently specific for prolactin, with there being no displacement of prolactin by insulin. Prolactin receptor affinity (K_a), as assessed by Scatchard analysis, was $8.7 \times 10^7 \text{ M}^{-1}$ with approximately 5.1×10^4 receptor sites/cell. This estimate of the association constant is lower than that obtained by Sakai *et al.* (1978) in enzymically-dissociated lactating mouse cells ($K_a = 1.1 \times 10^9 \text{ M}^{-1}$). The reason for this contrast in receptor affinity estimates is not clear, although values similar to that obtained here have been obtained in cells from virgin and pregnant mice ($K_a = 4.2 \times 10^8 \text{ M}^{-1}$) (Sakai *et al.*, 1978) and in the particulate fraction from lactating mouse mammary tissue ($K_a = 1.1 \times 10^8 \text{ M}^{-1}$) (Frantz *et al.*, 1974).

The low estimate of receptor affinity was not due to factors such as the initial quality, or proteolytic degradation, of the ligand utilised in this study. Prolactin from the same source bound the prolactin receptor in rabbit mammary membranes with high affinity ($K_a = 1.9 \times 10^9 \text{ M}^{-1}$) (Chapter 3). The presence of protease inhibitors in the binding assay ensured that the tracer's integrity was maintained. This was confirmed by trichloroacetic acid precipitation, indicating that the ligand's affinity for its receptor was not compromised by proteolytic degradation. Incomplete incorporation of ^{125}I into oPrl, as in this particular experiment, does not influence Scatchard analysis of the binding data (Brooks *et al.*, 1982).

The low estimate of receptor affinity in the present study is probably due, therefore, to an over-estimate of non-specific binding. This can result from the use of an excess concentration of unlabelled prolactin in estimating non-specific binding, with non-specific binding being proportional to prolactin's concentration in the assay (Brooks *et al.*, 1982). Although this error can be corrected (Chamness & McGuire, 1975), this was precluded by the limited number of data points in this analysis.

Cyanide ions were included in the binding assay to inhibit both energy-dependent uptake and degradation of occupied and unoccupied hormone receptors (Costlow & Hample, 1982a). This should have prevented receptor relocalisation following harvesting of the cells from the incubation medium, enabling differences in receptor localisation to be detected during the prolactin binding assay. This energy-depletion of the cells would be predicted to have increased surface prolactin binding by a factor of 1 to 2 (Costlow & Hample, 1982b). This can not completely explain the high estimate of receptor number that was obtained (5.1×10^4 sites/cell),

particularly when compared to the estimate of 700 sites/cell obtained by Sakai *et al* (1985). However, an under-estimate of receptor affinity resulting from high non-specific binding would result in an over-estimate of receptor number (Chamness & McGuire, 1975). Although it was not possible to assess prolactin receptor number accurately by Scatchard analysis, it was possible when specifically bound prolactin was expressed as a proportion of [¹²⁵I]-oPrl in the assay (Brooks *et al.*, 1982).

No attempt was made in the present experiments to assess whether the milk fractions affected prolactin receptor affinity. The 10-30 kDa goat whey fraction does not directly affect prolactin binding to its receptor (Chapter 4), with FIL-induced changes in prolactin binding *in vivo* being mediated entirely through an effect on receptor number (Chapter 4). It was assumed, therefore, that effects on prolactin binding in this study were mediated entirely through changes in receptor number, with receptor affinity not being affected.

A high proportion (80-90%) of prolactin receptors were present on the surface of the isolated cells and this value is similar to that obtained, using a similar procedure, in rat mammary tumour cells (Costlow & Hample, 1982a). This contrasts with the value obtained using mammary membranes prepared from bromocriptine-treated rabbits, where the majority of prolactin receptors were present intracellularly (Djiane *et al.*, 1981a). However this *in vivo* value is possibly inaccurate, for the bromocriptine-induced reduction in circulating prolactin concentration may affect the intracellular distribution of receptors as a consequence of effects on receptor internalisation (Costlow, 1986).

In the [¹²⁵I]-prolactin binding assay described here, prolactin would bind only to receptors which were not occupied by the endogenous hormone (Costlow, 1986). Therefore, the increase in prolactin binding by permeabilised cells during culture is likely to reflect either *de novo* receptor synthesis, or recycling of previously occupied receptors. However, the rapidity of prolactin receptor turnover (Costlow & Hample, 1982a) makes the latter unlikely.

That the 10-30 kDa goat whey fraction did not affect total prolactin receptor number implies that receptor synthesis was not inhibited. Although it is possible that total protein synthesis was not inhibited as a consequence of the low levels of the whey fraction used in this study (Rennison *et al.*, 1993), the marked inhibition of protein secretion by FIL in later experiments makes this unlikely. This would suggest

that receptor synthesis is regulated separately in the mammary cell and is not affected by FIL's inhibition of total protein synthesis (Rennison *et al.*, 1993). The apparent differential control of protein and receptor synthesis could explain the observed independence of milk yield and mammary prolactin receptor number seen in the goat following short-term unilateral changes in milking frequency (Chapter 5). It is also apparent that degradation of prolactin receptors was not stimulated by the 10-30 kDa goat whey fraction, although the effects of FIL on total prolactin receptor number *in vivo* are possibly mediated through receptor degradation (Chapter 4).

The 10-30 kDa goat whey fraction acted to relocate prolactin receptors from the cell-surface to intracellular sites. This effect was mediated, at least in part, by FIL which, in addition to reducing cell-surface prolactin receptor number, also inhibited protein secretion. These effects are consistent with FIL's role as a feedback inhibitor of lactation (Addey *et al.*, 1991b). It was not possible, however, to demonstrate that inhibition of prolactin binding and protein secretion by FIL was specific to this protein, since the control protein (fraction 5) also had inhibitory effects in this study. The effects of fraction 5, which does not inhibit casein secretion in rabbit mammary explants (Addey *et al.*, 1991b), may be explained by the use of a different species and experimental system in the present study. The effects of FIL and fraction 5 were not non-specific effects due to the presence of exogenous protein, for an excess of bovine serum albumin was also present in the incubation medium. Although a non-specific effect on the isolated cells arising from the presence of milk proteins cannot be dismissed, the effects of FIL on casein secretion in rabbit mammary explants are specific to this protein (Addey *et al.*, 1991b).

FIL is present in milk at a concentration of approximately 100 ng protein/ml, with fraction 5 present at 2.5 μg protein/ml (Dr. C.V.P. Addey, personal communication). The inhibition of milk secretion by FIL is dose-dependent (Rennison *et al.*, 1993) and if the same is assumed for fraction 5, the relative potency of FIL (per μg of protein) is at least 25 times greater than that of fraction 5.

The predominant protein species in fraction 5 is α -lactalbumin (Dr. C.V.P. Addey, personal communication), although other protein species may be present. It is possible that α -lactalbumin inhibits milk secretion in isolated mouse mammary cells, although it is more likely that a FIL-related protein, resolving together with α -lactalbumin, is the factor responsible for the demonstrated effects. This is supported

by evidence in bovine milk, where FIL-related proteins resolve separately by anion-exchange chromatography (Addey *et al.*, 1991a).

In contrast to the inhibition of prolactin binding and protein secretion effected by FIL from mid-lactation goat's milk, FIL prepared from late-lactation goat's milk (late October to mid November, week 33+ of lactation) had no consistent effect. Although a 3rd resolved milk fraction was present in the 10-30 kDa goat whey fraction at late lactation, its profile differed from that of FIL at mid-lactation. It is possible, therefore, that the chemical characteristics of FIL change in late-lactation, possibly resulting in the loss of inhibitory activity for that particular protein. These chemical changes may also result in FIL resolving in a different location, possibly explaining the reduction, although not significant, in lactose secretion and prolactin-binding by cells incubated with the 10-30 kDa goat whey fraction minus FIL. It has been reported that the marked reductions in goat milk yield during late lactation (Hart, 1975) are accompanied by a lack of responsiveness to frequent-milking in pregnant goats (Blatchford & Peaker, 1982), although the responsiveness to milking-frequency is maintained in non-pregnant goats (Henderson *et al.*, 1983). It is possible that the factors responsible for controlling milk secretion in the pregnant goat, in late-lactation, differ from those implicated in non-pregnant animals.

The FIL-induced reduction in cell-surface hormone-receptor number would lessen the cells' sensitivity to circulating hormone. It seems likely that this is a consequence of, rather than a cause of, the acute effects of FIL on protein secretion. The inhibition of protein secretion occurred in the absence of exogenous prolactin and over a period when the cells were refractory to prolactin. The reductions in cell-surface prolactin receptor number were therefore secondary to the effects of FIL on protein secretion. The reduced hormonal sensitivity mediated by FIL, whilst having no immediate role in controlling milk secretion is, however, likely to affect mammary cell differentiation, with long-term consequences for milk synthesis and secretion. Evidence for such chronic effects comes from the inhibition of mouse mammary cell differentiation by the 10-30 kDa goat whey fraction *in vitro* (Wilde *et al.*, 1991) and the correlation, in lactating mice, between mammary gland prolactin receptor number and litter weight gain (Sakai *et al.*, 1985).

FIL's inhibition of milk secretion is rapidly reversible (Wilde *et al.*, 1987a) and the same would be expected of the changes in surface hormone-receptor number if these are a consequence of the inhibition of secretion by FIL (Rennison *et al.*, 1993). A return of surface-receptor number to previous levels, on withdrawal of FIL, would allow hormonal resensitisation of the cells and would prevent a complete loss of the cells' differentiative state following milk removal. However, a reduction in total receptor number, as evidenced in the rabbit *in vivo* (Chapter 4), would have longer-term consequences.

Recent evidence suggests that FIL acts through the Golgi secretory apparatus to inhibit constitutive protein secretion (Ms M.E. Rennison, unpublished observation) and it is possible that the reduction in surface prolactin receptor number is mediated by this mechanism. Both occupied and unoccupied prolactin receptors are located in the Golgi secretory apparatus, as indicated by specific prolactin binding to a Golgi membrane fraction (Djiane *et al.*, 1981a). This, together with visualisation of internalised prolactin in the Golgi apparatus (Seddiki & Ollivier-Bousquet, 1991; Suard *et al.*, 1979), suggests that both internalised and newly-synthesised receptors are directed through the Golgi. An action of FIL on the Golgi could therefore inhibit the insertion of prolactin receptors into the cell-surface. This would result in a reduction in cell-surface prolactin-receptor number and an accumulation of receptors at intracellular sites, for receptor endocytosis is apparently independent of effects on the cytoskeleton (Djiane *et al.*, 1980). Such a mechanism of action would not be limiting to prolactin receptors alone, but would extend to all proteins, receptors or otherwise, which are normally directed through the Golgi prior to insertion into the cell surface. However, such a mechanism does not entirely explain the actions of FIL in this study, for there was no correlation of protein secretion and prolactin-binding by the isolated cells. This lack of correlation was best demonstrated by the different degrees of inhibition effected on each of these parameters by FIL and fraction 5. However, it is possible that this lack of correlation may be explained by temporal differences in the nature of measurements, with protein secretion reflecting a cumulative effect, whilst prolactin surface-receptor number may represent a snap-shot of the situation of that precise moment.

The absence of an effect of the FIL-containing 10-30 kDa goat whey fraction on total prolactin receptor number was in contrast to the effects on receptor number seen *in vivo* (Chapter 4). An effect of the 10-30 kDa goat whey fraction on total

receptor number may occur only when synthesised proteins are directed from secretory to degradative pathways. Such a mechanism for receptor down-regulation is supported by evidence in adipocytes, where the impaired recycling of insulin receptors shunts receptors to a chloroquine-sensitive degradative pathway (Marshall, 1985). Alternatively, an induction of intracellular degradation by the 10-30 kDa goat whey fraction could possibly be a consequence of mammary differentiation being reversed (Stewart *et al.*, 1988; Wilde *et al.*, 1989a), possibly through a reduced sensitivity to circulating hormones. The lack of an effect on total prolactin receptor number in the present study could then be explained by the apparent maintenance of the differentiated state in the isolated cells. An effect on total prolactin receptor number would then only be apparent when the cells started to de-differentiate and intracellular degradation was induced.

6.5 Conclusion

FIL acted within hours, in isolated lactating mammary cells, to internalise prolactin receptors from the cell-surface to intracellular sites. This reduced sensitivity to circulating hormones did not mediate the acute effects of FIL on milk secretion, which were independent of exogenous prolactin, but could be of importance in mediating long-term differentiative effects in the mammary gland.

CHAPTER SEVEN

SUMMARY AND DISCUSSION

At the start of this study, it was known that milk secretion by the mammary gland is controlled locally by a milk constituent (Wilde & Peaker, 1990), which had been identified as a milk protein and described as a feedback inhibitor of lactation (FIL; Addey *et al.*, 1991b). In addition, long-term alterations in milking frequency or efficiency were known to affect mammary differentiation (Wilde *et al.*, 1987b; Wilde *et al.*, 1989b) and this was accompanied by effects on mammary hormone receptor number (McKinnon *et al.*, 1988). Whether FIL mediated these effects on mammary differentiation and hormone receptor number was not known, and it was this question that I intended to address.

Initial investigations used the lactating rabbit, the steady rate of milk secretion over a long-suckling interval (Calvert *et al.*, 1987) and a prolactin-dependent lactation (Taylor & Peaker, 1975) making this species an ideal model. It was demonstrated that a significant reduction in mammary prolactin receptor number occurs at the end of the normal suckling interval, as a result of milk accumulation within the gland (Chapter 3). The reduction in receptor number preceded the locally-induced decrease in the rate of milk accumulation (Calvert *et al.*, 1987). This suggested that FIL, which had been shown to inhibit milk secretion in rabbit mammary tissue *in vitro* (Addey *et al.*, 1991b), may affect milk secretion through effects on mammary prolactin receptor number. The possibility of such a mechanism operating was investigated by intraductally injecting FIL into the rabbit mammary gland, with a reduction in both mammary prolactin and IGF-I receptor number being demonstrated (Chapter 4). It was established, therefore, that FIL not only regulates milk secretion but is also responsible, at least in part, for the reduction in hormone receptor number resulting from milk accumulation.

However, in the lactating goat, it was demonstrated that milk yield was not governed by prolactin receptor number alone. Effects on milk yield resulting from short-term unilateral alterations in milking frequency, thought to be mediated by FIL (Wilde & Peaker, 1990), were independent of mammary prolactin receptor number (Chapter 5). As it was likely that the hormonal sensitivity of the mammary gland was affected in this study and only hormone receptors present on the basal cell-surface are

exposed to the circulating hormone, it was thought that FIL may have affected the distribution of receptors in the milk secretory cell. This possibility was investigated using isolated lactating mouse mammary cells (Chapter 6). In this system, FIL acted within hours to relocate prolactin receptors from cell-surface to intracellular sites, without affecting total receptor number. This demonstrated that FIL could affect the distribution of prolactin receptors within the mammary cell and that the initial effects of FIL on mammary hormonal sensitivity may be mediated through alterations in cell-surface receptor number.

The mechanism by which FIL acts on the mammary gland has not been elucidated. It is believed that FIL acts via the apical surface of the mammary secretory cell (Henderson & Peaker, 1987), for a goat possessing a relatively small alveolar volume has a relatively high rate of milk secretion per unit mammary gland volume, by virtue of storing a greater proportion of milk of its milk at a site where FIL is inactive (Peaker & Blatchford, 1988). In addition, cows with proportionately large cisterns are more tolerant of once daily milking (Knight & Dewhurst, 1992) and less responsive to thrice daily milking (Dewhurst & Knight, 1992). An action of FIL across the apical membrane is supported by the effects that are observed following introduction of FIL into the mammary gland via the teat duct (Chapter 4; Wilde *et al.*, 1987; 1988a; 1988b).

It is not immediately apparent how a factor acting on the apical surface of the secretory cell could affect hormone receptors which are thought to be associated with the basal cell-surface (see Birkinshaw & Falconer (1972)). However, solubilisation of apical membrane isolated from mid-lactation goat's milk (Shennan, 1992a; 1992b) with 0.5% (w/v) CHAPS (Ashkenazi *et al.*, 1987) reveals high-affinity prolactin binding sites when assessed by radio-receptor assay, with polyethylene glycol 8000 precipitation (Cuatrecasas, 1972) of hormone-receptor complexes (E.J. Stewart, C.N. Bennett & C.H. Knight, unpublished observations). It can, therefore, be tentatively stated that receptors for prolactin are present on the apical membrane of the caprine milk secretory cell. This could have important implications in explaining the mechanism of FIL's effects on receptor distribution.

The study using isolated mouse mammary cells demonstrated that protein synthesis and secretion was independent of exogenous prolactin. On this basis, it is

unlikely that FIL-induced effects on receptor distribution account for FIL's inhibition of constitutive protein secretion (Rennison *et al.*, 1993). Instead, it suggests that effects on receptor distribution are secondary to FIL's inhibition of protein secretion.

FIL appears to disrupt the Golgi secretory apparatus whilst acting to affect protein secretion (Rennison *et al.*, 1993). This action on the Golgi provides a possible mechanism by which prolactin receptor distribution is affected as a consequence of FIL action. Both internalised and newly-synthesised prolactin receptors may be directed through the Golgi, since there is specific prolactin binding to a Golgi membrane fraction (Djiane *et al.*, 1981a) and internalised prolactin can be visualised in the Golgi apparatus (Seddiki & Ollivier-Bousquet, 1991; Suard *et al.*, 1979). Receptors present in the Golgi may be recycled to either the basal or apical cell-surfaces, or directed towards degradative pathways (Kelly *et al.*, 1984). An action of FIL prior to, or at, the *trans*-Golgi network, where the sorting of secretory proteins occurs (Rothman & Orci, 1992), could affect prolactin receptor distribution through an action on both apical and basal cell-trafficking, resulting in the accumulation of receptors in the Golgi. Alternatively, if it is assumed that prolactin receptors are cycled between the apical and basal surfaces, as is suggested by the presence of receptors on the apical surface, an action of FIL on apical cell-trafficking only could affect basal prolactin receptor number.

This FIL-induced inhibition of cellular-trafficking would, at some point, have to affect total hormone receptor number if the reduction in receptor number induced by FIL in lactating rabbit mammary glands is to be explained (Chapter 4). This study revealed differences between species in the rapidity of FIL action on total receptor number and in the relationship of these effects to changes in the rate of milk accumulation. In the rabbit, the changes in receptor number appear to precede changes in the rate of milk accumulation, although the rate of milk secretion may have been affected at this time. In the goat, the effects of milking-frequency on milk yield were independent of total receptor number, but possibly dependent on receptor distribution. These differences may reflect contrasting lactational requirements for circulating prolactin, with prolactin being the major galactopoietic hormone in the rabbit (Taylor & Peaker, 1975) whilst having a relatively minor role in the goat, acting to modulate rather than dictate the milk yield of individual glands (Knight *et al.*, 1990a).

The FIL-induced reduction in hormone receptor number does not appear to be mediated through an effect on receptor synthesis, for although total protein synthesis is inhibited by FIL (Rennison *et al.*, 1993), the 10-30 kDa goat whey fraction does not affect total receptor number in isolated lactating mouse mammary cells. An absence of a direct effect of FIL on receptor synthesis is supported further by the demonstration that receptor number is not affected by the increased rate of milk synthesis resulting from short-term (8 days) more-frequent milking of goat mammary glands. Similarly, it appears that FIL does not automatically shunt receptors towards a degradative pathway (Marshall, 1985), for this would have resulted in a reduction in total receptor number in 10-30 kDa whey fraction treated mammary cells and in less-frequently milked goat mammary glands.

Another mechanism is likely to be operating, therefore, to explain the effects of FIL on hormone receptor number observed *in vivo*. It is possible that receptor number is affected only when the differentiative state of the gland is reversed as a consequence of FIL action. De-differentiation of the rabbit mammary gland can be induced rapidly by the FIL-containing 10-30 kDa goat whey fraction *in vivo* (Wilde *et al.*, 1988b) and this is possibly a consequence of reduced hormonal sensitivity resulting from a FIL-induced decrease in cell-surface receptor number. The suggestion that total receptor number is not affected until de-differentiation has occurred is supported by the absence of an effect on total receptor number when mammary differentiation is apparently maintained (eg in isolated mouse mammary cells and in goat mammary glands subject to short-term alterations in milking frequency). In contrast, an effect on total receptor number is apparent when an effect on differentiation is known to have occurred (Hayden & Smith, 1981; McKinnon *et al.*, 1988) or would be predicted (eg following milk accumulation, or FIL-introduction, in the rabbit mammary gland).

Intracellular degradation of casein appears to be inversely related to the differentiative state of the mammary tissue (Stewart *et al.*, 1988; Wilde *et al.*, 1989a). The effect of milk accumulation or FIL on receptor number may only occur, therefore, when intracellular degradation of receptors is induced by tissue de-differentiation. This mechanism of action would not be limiting to prolactin receptors alone, but would also extend to other hormone receptors (as was demonstrated for IGF-I receptors). A long-term effect on prolactin receptor number may also result from a lessening of prolactin's up-regulation of its own receptor (Kelly *et al.*, 1984)

as a consequence of the FIL-induced reduction in mammary hormonal sensitivity. The process by which long-term changes in receptor number result from FIL action is an area requiring further investigation.

In conclusion, this study demonstrated that FIL is responsible for changes in mammary prolactin receptor number and distribution resulting from milk accumulation and alterations in milking frequency or efficiency. It is likely that this modulation of mammary hormonal sensitivity is not involved in immediate control of milk secretion rate, but is responsible for long-term effects on milk yield and mammary differentiation. This provides a mechanism whereby the supply of maternal milk can be matched to the needs of the neonate.

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